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PCT

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification⁶: C12N 15/11, 9/00, A61K 48/00, 31/70, C07H 21/00	A1	(11) International Publication Number: WO 97/37014 (43) International Publication Date: 9 October 1997 (09.10.97)
(21) International Application Number: PCT/GB97/00929 (22) International Filing Date: 2 April 1997 (02.04.97) (30) Priority Data: 9606961.2 2 April 1996 (02.04.96) GB (71) Applicant (for all designated States except US): PROVOST, FELLOWS AND SCHOLARS OF THE COLLEGE OF THE HOLY AND UNDIVIDED TRINITY OF QUEEN ELIZABETH NEAR DUBLIN [IE/IE]; Trinity College Dublin, Dublin 2 (IE). (72) Inventors; and (75) Inventors/Applicants (for US only): FARRAR, Gwenth, Jane [IE/IE]; 9 The Crescent, Monkstown D20, County Dublin (IE). HUMPHRIES, Peter [GB/IE]; 5 Holmwood, Cabinteely D15, County Dublin (IE). KENNA, Paul, Francis [IE/IE]; 176 New Cabra Road, Dublin 7 (IE). (74) Agent: MURGITROYD & COMPANY; 373 Scotland Street, Glasgow G5 8QA (GB).	(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the</i> <i>claims and to be republished in the event of the receipt of</i> <i>amendments.</i>	
(54) Title: GENETIC SUPPRESSION AND REPLACEMENT		
(57) Abstract <p>A strategy for suppressing specifically or partially specifically an endogenous gene and introducing a replacement gene, said strategy comprising the steps of: 1) providing suppressing nucleic acids or other suppression effectors able to bind to an endogenous gene, gene transcript or gene product to be suppressed, and 2) providing genomic DNA or cDNA (complete or partial) encoding a replacement gene wherein the suppressing nucleic acids are unable to bind to equivalent regions in the genomic DNA or cDNA to prevent expression of the replacement gene. The replacement nucleic acids have modifications in one or more third base (wobble) positions such that replacement nucleic acids still code for the wild type or equivalent amino acids.</p>		

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1 **"Genetic Suppression and Replacement"**

2

3 The present invention relates to a strategy for
4 suppressing a gene. In particular the invention
5 relates to suppression of mutated genes which give rise
6 to a dominant or deleterious effect, either
7 monogenically or polygenically. The invention relates
8 to a strategy for suppressing a gene or disease allele
9 using methods which do not target the disease allele
10 specifically but instead can be targeted towards a
11 broad range of sequences in a particular gene. A
12 particular embodiment of the invention is the use of
13 suppression strategies to target either the disease or
14 normal alleles alone or to target both disease and
15 normal alleles. A further embodiment of the invention
16 is the use of the wobble hypothesis to enable continued
17 expression of a replacement normal or beneficial gene
18 (a gene modified from the wild type such that it
19 provides an additional beneficial effect(s)). The
20 replacement gene will have nucleotide changes from the
21 endogenous wild type gene but will code for identical
22 amino acids as the wild type gene. The strategy
23 circumvents the need for a specific therapy for every
24 mutation within a given gene. In addition the
25 invention allows greater flexibility in choice of

1 target sequence for suppression of a disease allele.

2

3 The invention also relates to a medicament or
4 medicaments for use in suppressing a deleterious allele
5 which is present in a genome of one or more individuals
6 or animals.

7

8 Generally the present invention will be useful where
9 the gene, which is naturally present in the genome of a
10 patient, contributes to a disease state. Generally,
11 one allele of the gene in question will be mutated,
12 that is, will possess alterations in its nucleotide
13 sequence that affects the function or level of the gene
14 product. For example, the alteration may result in an
15 altered protein product from the wild type gene or
16 altered control of transcription and processing.
17 Inheritance or somatic acquisition of such a mutation
18 can give rise to a disease phenotype or can predispose
19 an individual to a disease phenotype. However the gene
20 of interest could also be of wild type phenotype, but
21 contribute to a disease state in another way such that
22 the suppression of the gene would alleviate or improve
23 the disease state or improve the effectiveness of an
24 administered therapeutic compound.

25

26 Generally, suppression effectors such as nucleic acids
27 - antisense or sense, ribozymes, peptide nucleic acids
28 (PNAs), triple helix forming oligonucleotides, peptides
29 and /or antibodies directed to sequences in a gene, in
30 transcripts or in protein, can be employed in the
31 invention to achieve gene suppression.

32

33 BACKGROUND

34

35 Studies of degenerative hereditary ocular conditions,
36 including Retinitis Pigmentosa (RP) and various macular

1 dystrophies have resulted in a substantial elucidation
2 of the molecular basis of these debilitating human
3 retinal degenerations. Applying the approach of
4 genetic linkage, x-linked RP (xLRP) genes have been
5 localised to the short arm of the X chromosome (Ott et
6 al. 1990) - subsequently the gene involved in one form
7 of xLRP has been identified. Various genes involved in
8 autosomal dominant forms of RP (adRP) have been
9 localised. The first of these mapped on 3q close to
10 the gene encoding the rod photoreceptor protein
11 rhodopsin (McWilliam et al. 1989; Dryja et al. 1990).
12 Similarly, an adRP gene was placed on 6p close to the
13 gene encoding the photoreceptor protein peripherin
14 (Farrar et al. 1991a,b; Kajiwara et al. 1991).
15 Other adRP genes have been mapped to discrete
16 chromosomal locations however the disease genes as yet
17 remain uncharacterised. As in xLRP and adRP, various
18 genes involved in autosomal recessive RP (arRP) have
19 been localised and in some cases molecular defects
20 characterised (Humphries et al. 1992; Farrar et al.
21 1993; Van Soest et al. 1994). Similarly a number of
22 genes involved in macular dystrophies have been mapped
23 (Mansergh et al. 1995). Genetic linkage, together with
24 techniques for mutational screening of candidate genes,
25 enabled identification of causative dominant mutations
26 in the genes encoding rhodopsin and peripherin.
27 Globally about 100 rhodopsin mutations have been found
28 in patients with RP or congenital stationary night
29 blindness. Similarly approximately 40 mutations have
30 been characterised in the peripherin gene in patients
31 with RP or macular dystrophies. Knowledge of the
32 molecular aetiology of these retinopathies has
33 stimulated the generation of animal models and the
34 exploration of methods of therapeutic intervention
35 (Farrar et al. 1995; Humphries et al. 1997).
36

1 Similar to RP, osteogenesis imperfecta (OI) is an
2 autosomal dominantly inherited human disorder whose
3 molecular pathogenesis is extremely genetically
4 heterogeneous. OI is often referred to as 'brittle
5 bone disease' although additional symptoms including
6 hearing loss, growth deficiency, bruising, loose
7 joints, blue sclerae and dentinogenesis imperfecta are
8 frequently observed (McKusick, 1972). Mutations in the
9 genes encoding the two type I collagen chains (collagen
10 1A1 and 1A2) comprising the type I collagen heterodimer
11 have been implicated in OI. Indeed hundreds of
12 dominantly acting mutations have been identified in OI
13 patients in these two genes, many of which are single
14 point mutations, although a number of insertion and
15 deletion mutations have been found (Willing et al.
16 1993; Zhuang et al. 1996). Similarly mutations in
17 these genes have also been implicated in Ehlers-Danlos
18 and Marfan syndromes (Dalglish et al. 1986; Phillips
19 et al. 1990; D'Alessio et al. 1991; Vasan NS et al.
20 1991).

21
22 Generally, gene therapies utilising viral and non-viral
23 delivery systems have been used to treat inherited
24 disorders, cancers and infectious diseases. However,
25 many studies have focused on recessively inherited
26 disorders, the rationale being that introduction and
27 expression of the wild type gene may be sufficient to
28 prevent/ameliorate the disease phenotype. In contrast
29 gene therapy for dominant disorders will require
30 suppression of the dominant disease allele. Notably
31 many of the characterised mutations causing inherited
32 diseases such as RP or OI are inherited in an autosomal
33 dominant fashion. Indeed there are over 1,000
34 autosomal dominantly inherited disorders in man. In
35 addition there are many polygenic disorders due to
36 co-inheritance of a number of genetic components

1 which together give rise to the disease state.
2 Effective gene therapies for dominant or polygenic
3 diseases may be targeted to the primary defect and in
4 this case may require suppression of the disease allele
5 while in many cases still maintaining the function of
6 the normal allele. This will be particularly relevant
7 where disease pathology is due to a gain of function
8 mutation rather than due to reduced levels of wild type
9 protein. Alternatively suppression therapies may be
10 targeted to secondary effects associated with the
11 disease pathology: one example is programmed cell death
12 (apoptosis) which has been observed in many inherited
13 disorders.

14
15 Strategies to differentiate between normal and disease
16 alleles and to selectively switch off the disease
17 allele using suppression effectors inter alia antisense
18 DNA/RNA, PNAs, ribozymes or triple helix DNA, targeted
19 towards the disease mutation may be difficult in many
20 cases - frequently disease and normal alleles differ
21 by only a single nucleotide. A further difficulty
22 inhibiting development of gene therapies is the
23 heterogeneous nature of some dominant disorders - many
24 different mutations in the same gene give rise to a
25 similar disease phenotype. Development of specific
26 gene therapies for each of these may be prohibitive in
27 terms of cost. To circumvent difficulties associated
28 with specifically targeting the disease mutation and
29 with the genetic heterogeneity present in inherited
30 disorders, a novel strategy for gene suppression and
31 gene replacement exploiting the degeneracy of the
32 genetic code, thereby allowing flexibility in choice of
33 target sequence for suppression and providing a means
34 of gene suppression which is independent of the disease
35 mutation, is described in the invention.

36

1 PRIOR ART

2

3 Suppression effectors have been used previously to
4 achieve specific suppression of gene expression.
5 Antisense DNA and RNA has been used to inhibit gene
6 expression in many instances. Modifications, such as
7 phosphorothioates, have been made to oligonucleotides
8 to increase resistance to nuclease degradation, binding
9 affinity and uptake (Cazenave et al. 1989; Sun et al.
10 1989; McKay et al. 1996; Wei et al. 1996). In some
11 instances, using antisense and ribozyme suppression
12 strategies has led to reversal of a tumour phenotype by
13 reducing expression of a gene product or by cleaving a
14 mutant transcript at the site of the mutation (Carter
15 and Lemoine 1993; Lange et al. 1993; Valera et al.
16 1994; Dosaka-Akita et al. 1995; Feng et al. 1995;
17 Quattrone et al. 1995; Ohta et al. 1996). For example,
18 neoplastic reversion was obtained using a ribozyme
19 targeted to a H-ras mutation in bladder carcinoma cells
20 (Feng et al. 1995). Ribozymes have also been proposed
21 as a means of both inhibiting gene expression of a
22 mutant gene and of correcting the mutant by targeted
23 trans-splicing (Sullenger and Cech 1994; Jones et al.
24 1996). Ribozymes can be designed to elicit
25 autocatalytic cleavage of RNA targets, however, the
26 inhibitory effect of some ribozymes may be due in part
27 to an antisense effect due to the antisense sequences
28 flanking the catalytic core which specify the target
29 site (Ellis and Rodgers 1993; Jankowsky and Schwenzer
30 1996). Ribozyme activity may be augmented by the use
31 of, for example, non-specific nucleic acid binding
32 proteins or facilitator oligonucleotides (Herschlag et
33 al. 1994; Jankowsky and Schwenzer 1996). Multitarget
34 ribozymes (connected or shotgun) have been suggested as
35 a means of improving efficiency of ribozymes for gene
36 suppression (Ohkawa et al. 1993). Triple helix

1 approaches have also been investigated for sequence
2 specific gene suppression - triplex forming
3 oligonucleotides have been found in some cases to bind
4 in a sequence specific manner (Postel et al. 1991;
5 Duval-Valentin et al. 1992; Hardenbol and Van Dyke
6 1996; Porumb et al. 1996). Similarly peptide nucleic
7 acids have been shown to inhibit gene expression
8 (Hanvey et al. 1992; Knudson and Nielsen 1996; Taylor
9 et al. 1997). Minor groove binding polyamides can bind
10 in a sequence specific manner to DNA targets and hence
11 may represent useful small molecules for future
12 suppression at the DNA level (Trauger et al. 1996). In
13 addition, suppression has been obtained by interference
14 at the protein level using dominant negative mutant
15 peptides and antibodies (Herskowitz 1987; Rimsky et al.
16 1989; Wright et al. 1989). In some cases suppression
17 strategies have lead to a reduction in RNA levels
18 without a concomitant reduction in proteins, whereas in
19 others, reductions in RNA have been mirrored by
20 reductions in protein.

21
22 There is now an armament with which to obtain gene
23 suppression. This, in conjunction with a better
24 understanding of the molecular etiology of disease,
25 results in an ever increasing number of disease targets
26 for therapies based on suppression. In many cases,
27 complete suppression of gene expression has been
28 difficult to achieve. Possibly a combined approach
29 using a number of suppression effectors may aid in
30 this. For some disorders it may be necessary to block
31 expression of a disease allele completely to prevent
32 disease symptoms whereas for others low levels of
33 mutant protein may be tolerated. In parallel with an
34 increased knowledge of the molecular defects causing
35 disease has been the realisation that many disorders
36 are genetically heterogeneous. Examples in which

1 multiple genes and/or multiple mutations within a gene
2 can give rise to a similar disease phenotype include
3 osteogenesis imperfecta, familial hypercholesterolemia,
4 retinitis pigmentosa, and many others. The utility of
5 the degeneracy of the genetic code (wobble hypothesis)
6 to enable suppression of one or both alleles of a gene
7 and the introduction of a replacement gene such that
8 it escapes suppression has been exploited in the
9 invention.

10
11 The invention addresses shortcomings of the prior art
12 by providing a novel approach to the design of
13 suppression effectors directed to target alleles of a
14 gene carrying a deleterious mutation. Suppression of
15 every mutation giving rise to a disease phenotype may
16 be costly and problematic. Disease mutations are
17 often single nucleotide changes. As a result
18 differentiating between the disease and normal alleles
19 may be difficult. Some suppression effectors require
20 specific sequence targets, for example, hammerhead
21 ribozymes cleave at NUX sites and hence may not be able
22 to target many mutations. Notably, the wide spectrum
23 of mutations observed in many diseases adds additional
24 complexity to the development of therapeutic strategies
25 for such disorders - some mutations may occur only once
26 in a single patient. A further problem associated with
27 suppression is the high level of homology present in
28 coding sequences between members of some gene families.
29 This can limit the range of target sites for
30 suppression which will enable specific suppression of a
31 single member of such a gene family.

32
33 The present invention circumvents shortcomings in the
34 prior art by utilising the degeneracy of the genetic
35 code. In the invention suppression effectors are
36 designed specifically to sequences in coding regions of

1 genes or in gene products. Typically one allele of the
2 gene contains a mutation with a deleterious effect.
3 Suppression targeted to coding sequences provides
4 greater flexibility in choice of target sequence for
5 suppression in contrast to suppression directed towards
6 single disease mutations. Additionally the invention
7 provides for the introduction of a replacement gene
8 with modified sequences such that the replacement gene
9 is protected from suppression. The replacement gene is
10 modified at third base wobble positions and hence
11 provides the wild type gene product. Notably, the
12 invention has the advantage that the same suppression
13 strategy could be used to suppress, in principle, many
14 mutations in a gene. This is particularly relevant
15 when large numbers of mutations within a single gene
16 cause disease pathology. The replacement gene provides
17 (when necessary) expression of the normal protein
18 product when required to ameliorate pathology
19 associated with reduced levels of wild type protein.
20 The same replacement gene could in principle be used in
21 conjunction with the suppression of many different
22 disease mutations within a given gene. Target
23 sequences may be found in any part of the coding
24 sequence. Suppression in coding sequence holds the
25 advantage that such sequences are present in both
26 precursor and mature RNAs, thereby enabling suppressors
27 to target all forms of RNA.

28
29 In summary the invention involves gene suppression of
30 disease and normal alleles targeting coding sequences
31 in a gene and when necessary gene replacement such
32 that the replacement gene cannot be suppressed.
33 Replacement genes are modified at third base positions
34 (wobble positions) so that they code for the correct
35 amino acids but are protected completely or partially
36 from suppression. The same suppression and replacement

1 steps can be used for many disease mutations in a given
2 gene. Suppression and replacement can be undertaken in
3 conjunction with each other or separately.
4

5
6 DESCRIPTION OF INVENTION
7

8 According to the present invention there is provided a
9 strategy for suppressing expression of an endogenous
10 gene with a deleterious mutation, wherein said strategy
11 comprises providing suppression effectors such as
12 antisense nucleic acids able to bind to sequences of a
13 gene to be suppressed, to prevent the functional
14 expression thereof.
15

16 Generally, the term suppression effectors means nucleic
17 acids, peptide nucleic acids (PNAs), peptides,
18 antibodies or modified forms of these used to silence
19 or reduce gene expression in a sequence specific
20 manner.
21

22 Suppression effectors, such as antisense nucleic acids
23 can be DNA or RNA, can typically be directed to coding
24 sequence however suppression effectors can be targeted
25 to coding sequence and can also be targetted to 5'
26 and/or 3' untranslated regions and/or introns and/or
27 control regions and/or sequences adjacent to a gene or
28 to any combination of such regions of a gene.

29 Antisense nucleic acids including both coding and
30 non-coding sequence can be used if required to help to
31 optimise suppression. Binding of the suppression
32 effector(s) prevents or lowers functional expression of
33 the endogenous gene.
34

35 Generally the term 'functional expression' means the
36 expression of a gene product able to function in a

1 manner equivalent to or better than a wild type
2 product. In the case of a mutant gene or predisposing
3 gene 'functional expression' means the expression of a
4 gene product whose presence gives rise to a deleterious
5 effect or predisposes to a deleterious effect. By
6 deleterious effect is meant giving rise to or
7 predisposing to disease pathology or altering the
8 effect(s) and/or efficiency of an administered
9 compound.

10

11 In a particular embodiment of the invention the
12 strategy further employs ribozymes which can be
13 designed to elicit cleavage of target RNAs. The
14 strategy further employs nucleotides which form triple
15 helix DNA. The strategy can employ peptide nucleic
16 acids for suppression. Nucleic acids for antisense,
17 ribozymes, triple helix and peptide nucleic acids may
18 be modified to increase stability, binding efficiencies
19 and uptake (see prior art). Nucleic acids can be
20 incorporated into a vector. Vectors include naked DNA,
21 DNA plasmid vectors, RNA or DNA virus vectors, lipids,
22 polymers or other derivatives and compounds to aid gene
23 delivery and expression.

24

25 The invention further provides the use of antisense
26 nucleotides, ribozymes, PNAs, triple helix nucleotides
27 or other suppression effectors alone or in a vector or
28 vectors, wherein the nucleic acids are able to bind
29 specifically or partially specifically to coding
30 sequences of a gene to prevent or reduce the functional
31 expression thereof, in the preparation of a medicament
32 for the treatment of an autosomal dominant or polygenic
33 disease or to increase the utility and/or action of an
34 administered compound.

35

36 In a further embodiment of the invention target

1 sequences for suppression can include non-coding
2 sequences of the gene.

3

4 According to the present invention there is provided a
5 strategy for suppressing specifically or partially
6 specifically an endogenous gene and (if required)
7 introducing a replacement gene, said strategy
8 comprising the steps of:

9

- 10 1. providing nucleic acids or other suppression
11 effectors able to bind to an endogenous gene, gene
12 transcript or gene product to be suppressed and
13
- 14 2. providing genomic DNA or cDNA (complete or
15 partial) encoding a replacement gene wherein the
16 nucleic acids are unable to bind to equivalent
17 regions in the genomic DNA or cDNA to prevent
18 expression of the replacement gene. The
19 replacement nucleic acids will not be recognised
20 by suppression nucleic acids or will be recognised
21 less effectively than the endogenous gene. The
22 coding sequence of replacement nucleic acids can
23 be altered to prevent or reduce efficiency of
24 suppression. Replacement nucleic acids have
25 modifications in one or more third base (wobble)
26 positions such that replacement nucleic acids
27 still code for the wild type or equivalent amino
28 acids.

29

30 In a particular embodiment of the invention there is
31 provided a strategy for gene suppression targeted to
32 coding sequences of the gene to be suppressed.
33 Suppression will be specific or partially specific to
34 one allele, for example, to the allele carrying a
35 deleterious mutation. Suppressors are targeted to
36 coding regions of a gene or to a combination of coding

1 and non-coding regions of a gene. Suppressors can be
2 targeted to a characteristic of one allele of a gene
3 such that suppression is specific or partially specific
4 to one allele of a gene (PCT/GB97/00574). The
5 invention further provides for use of replacement
6 nucleic acids with altered coding sequences such that
7 replacement nucleic acids will not be recognised (or
8 will be recognised less effectively) by suppression
9 nucleic acids which are targeted specifically or
10 partially specifically to one allele of the gene to be
11 suppressed. Replacement nucleic acids provide the wild
12 type gene product, an equivalent gene product or an
13 improved gene product but are protected completely or
14 partially from suppression effectors targeted to coding
15 sequences.

16
17 In a further embodiment of the invention replacement
18 nucleic acids are provided such that replacement
19 nucleic acids will not be recognised by naturally
20 occurring suppressors found to inhibit or reduce gene
21 expression in one or more individuals, animals or
22 plants. The invention provides for use of replacement
23 nucleic acids which have altered sequences targeted by
24 suppressors of the gene such that suppression by
25 naturally occurring suppressors is completely or
26 partially prevented.

27
28 In an additional embodiment of the invention there is
29 provided replacement nucleic acids with altered
30 nucleotide sequence in coding regions such that
31 replacement nucleic acids code for a product with one
32 or more altered amino acids. Replacement nucleic acids
33 provide a gene product which is equivalent to
34 or improved compared with the naturally occurring
35 endogenous wild type gene product.

36

1 In an additional embodiment of the invention there is
2 provided a strategy to suppress a gene where the gene
3 transcript or gene product interferes with the action
4 of an administered compound.

5
6 The invention further provides the use of a vector or
7 vectors containing suppression effectors in the form of
8 nucleic acids, said nucleic acids being directed
9 towards coding sequences or combinations of coding and
10 non-coding sequences of the target gene and vector(s)
11 containing genomic DNA or cDNA encoding a replacement
12 gene sequence to which nucleic acids for suppression
13 are unable to bind (or bind less efficiently), in the
14 preparation of a combined medicament for the treatment
15 of an autosomal dominant or polygenic disease. Nucleic
16 acids for-suppression or replacement gene nucleic acids
17 may be provided in the same vector or in separate
18 vectors. Nucleic acids for suppression or replacement
19 gene nucleic acids may be provided as a combination of
20 nucleic acids alone or in vectors.

21
22 The invention further provides a method of treatment
23 for a disease caused by an endogenous mutant gene, said
24 method comprising sequential or concomitant
25 introduction of

26
27 (a) nucleic acids to the coding regions of a gene to
28 be suppressed and/or nucleic acids to coding
29 regions and any combination of 5' and/or 3'
30 untranslated regions, intronic regions, control
31 regions or regions adjacent to a gene to be
32 suppressed

33
34 (b) replacement nucleic acids with sequences which
35 allow the replacement gene to be expressed.

36

1 The nucleic acid for gene suppression can be
2 administered before, after or at the same time as the
3 replacement gene is administered.

4
5 The invention further provides a kit for use in the
6 treatment of a disease caused by a deleterious mutation
7 in a gene, the kit comprising nucleic acids for
8 suppression able to bind to the gene to be suppressed
9 and if required a replacement nucleic acid to replace
10 the mutant gene having sequence which allows it to be
11 expressed and completely or partially escape
12 suppression.

13
14 Nucleotides can be administered as naked DNA or RNA.
15 Nucleotides can be delivered in vectors. Naked nucleic
16 acids or nucleic acids in vectors can be delivered with
17 lipids or other derivatives which aid gene delivery.
18 Nucleotides may be modified to render them more stable,
19 for example, resistant to cellular nucleases while
20 still supporting RNaseH mediated degradation of RNA or
21 with increased binding efficiencies (see prior art).
22 Antibodies or peptides can be generated to target the
23 protein product from the gene to be suppressed.

24
25 The strategy described herein has applications for
26 alleviating autosomal dominant diseases. Complete
27 silencing of a disease allele may be difficult to
28 achieve using antisense, PNA, ribozyme and triple helix
29 approaches or any combination of gene silencing
30 approaches. However small quantities of mutant product
31 may be tolerated in some autosomal dominant disorders.
32 In others a significant reduction in the proportion of
33 mutant to normal product may result in an amelioration
34 of disease symptoms. Hence this invention may be
35 applied to any autosomal dominantly or polygenically
36 inherited disease in man where the molecular basis of

1 the disease has been established or is partially
2 understood. This strategy will enable the same therapy
3 to be used to treat a range of different disease
4 mutations within the same gene. The development of
5 such approaches will be important to future therapies
6 for autosomal dominant and polygenic diseases, the key
7 to a general strategy being that it circumvents the
8 need for a specific therapy for every mutation causing
9 or predisposing to a disease. This is particularly
10 relevant in some disorders, for example, rhodopsin
11 linked autosomal dominant RP, in which to date about
12 one hundred different mutations in the rhodopsin gene
13 have been observed in adRP patients. Likewise hundreds
14 of mutations have been identified in the human type I
15 Collagen 1A1 and 1A2 genes in autosomal dominant
16 osteogenesis imperfecta. Costs of developing therapies
17 for each mutation are prohibitive at present.
18 Inventions such as this using a general approach for
19 therapy will be required. General approaches may be
20 targeted to the primary defect as is the case with this
21 invention or to secondary effects such as apoptosis.

22
23 This invention may be applied in gene therapy
24 approaches for biologically important polygenic
25 disorders affecting large proportions of the world's
26 populations such as age related macular degeneration,
27 glaucoma, manic depression, cancers having a familial
28 component and indeed many others. Polygenic diseases
29 require inheritance of more than one mutation
30 (component) to give rise to the disease state. Notably
31 an amelioration in disease symptoms may require
32 reduction in the presence of only one of these
33 components, that is, suppression of one genotype which,
34 together with others leads to the disease phenotype,
35 may be sufficient to prevent or ameliorate symptoms of
36 the disease. In some cases suppression of more than

1 one component may be required to improve disease
2 symptoms. This invention may be applied in possible
3 future interventive therapies for common polygenic
4 diseases to suppress a particular genotype(s) using
5 suppression and when necessary replacement steps.
6

7 The present invention is exemplified using four genes:
8 human rhodopsin, mouse rhodopsin, human peripherin and
9 human collagen 1A2. The first of these genes are
10 retinal specific. In contrast, collagen 1A2 is
11 expressed in a range of tissues including skin and
12 bone. While these four genes have been used as
13 examples there is no reason why the invention could not
14 be deployed in the suppression of many other genes in
15 which mutations cause or predispose to a deleterious
16 effect. Many examples of mutant genes which give rise
17 to disease phenotypes are available from the prior art
18 - these genes all represent targets for the invention.
19 The present invention is exemplified using hammerhead
20 ribozymes with antisense arms to elicit RNA cleavage.
21 There is no reason why other suppression effectors
22 directed towards genes, gene transcripts or gene
23 products could not be used to achieve gene suppression.
24 Many examples from the prior art detailing use of
25 suppression effectors inter alia antisense RNA/DNA,
26 triple helix, PNAs and peptides to achieve suppression
27 of gene expression are reported (see prior art).
28 The present invention is exemplified using hammerhead
29 ribozymes with antisense arms to elicit sequence
30 specific cleavage of transcripts from genes implicated
31 in dominant disorders and non-cleavage of transcripts
32 from replacement genes containing sequence
33 modifications in wobble positions such that the
34 replacement gene still codes for wild type protein.
35 The present invention is exemplified using suppression
36 effectors targeting sites in coding regions of the

1 human and mouse rhodopsin, human peripherin and human
2 collagen 1A2 genes. Target sites which include
3 sequences from transcribed but untranslated regions of
4 genes, introns, regions involved in the control of gene
5 expression, regions adjacent to the gene or any
6 combination of these could be used to achieve gene
7 suppression. Multiple suppression effectors, for
8 example, shotgun ribozymes could be used to optimise
9 efficiency of suppression when necessary.
10 Additionally, when required expression of a modified
11 replacement gene such that the replacement gene product
12 is altered from the wild type product such that it
13 provides a beneficial effect may be used to provide
14 gene product.

15

16

17 MATERIALS and METHODS

18

19

20 Cloning vectors

21

22 cDNA templates and ribozymes were cloned into
23 commercial expression vectors (pCDNA3, pZeoSV or
24 pBluescript) which enable expression in a test tube
25 from T7, T3 or SP6 promoters or expression in mammalian
26 cells from CMV or SV40 promoters. Inserts were placed
27 into the multiple cloning site (MCS) of these vectors
28 typically at or near the terminal ends of the MCS to
29 delete most of the MCS and thereby prevent any possible
30 problems with efficiency of expression subsequent to
31 cloning.

32

33 Sequencing protocols

34

35 Clones containing template cDNAs and ribozymes were
36 sequenced by ABI automated sequencing machinery using

1 standard protocols.

2

3 Expression of RNAs

4

5 RNA was obtained from clones *in vitro* using a
6 commercially available Ribomax expression system
7 (Promega) and standard protocols. RNA purifications
8 were undertaken using the Bio-101 RNA purification kit
9 or a solution of 0.3M sodium acetate and 0.2% SDS after
10 isolation from polyacrylamide gels. Cleavage reactions
11 were performed using standard protocols with varying
12 $MgCl_2$ concentrations (0-15mM) at 37°C, typically for 3
13 hours. Time points were performed at the predetermined
14 optimal $MgCl_2$ concentrations for up to 5 hours.

15 Radioactively labelled RNA products were obtained by
16 incorporating α - P^{32} rUTP (Amersham) in the expression
17 reactions (Gaughan et al. 1995). Labelled RNA products
18 were run on polyacrylamide gels before cleavage
19 reactions were undertaken for the purpose of RNA
20 purification and subsequent to cleavage reactions to
21 establish if RNA cleavage had been achieved. Cleavage
22 reactions were undertaken with 5mM Tris-HCl pH8.0 and
23 varying concentrations of $MgCl_2$ at 37°C.

24

25 RNA secondary structures

26

27 Predictions of the secondary structures of human and
28 mouse rhodopsin, human peripherin and human collagen
29 1A2 mRNAs were obtained using the RNAPlotFold program.
30 Ribozymes and antisense were designed to target areas
31 of the RNA that were predicted to be accessible to
32 suppression effectors, for example open loop
33 structures. The integrity of open loop structures was
34 evaluated from the 10 most probable RNA structures.
35 Additionally, predicted RNA structures for truncated
36 RNA products were generated and the integrity of open

1 loops between full length and truncated RNAs compared.

2

3 TEMPLATES and RIBOZYMES

4

5 Human Rhodopsin

6

7 Template cDNA

8 The human rhodopsin cDNA was cloned into the HindIII
9 and EcoRI sites of the MCS of pCDNA3 in a 5' to 3'
10 orientation allowing subsequent expression of RNA from
11 the T7 or CMV promoter in the vector. The full length
12 5'UTR sequence was inserted into this clone using
13 primer driven PCR mutagenesis and a HindIII (in pCDNA3)
14 to BstEII (in the coding sequence of the human
15 rhodopsin cDNA) DNA fragment (Sequence 1).

16

17 cDNA with altered sequence at a wobble position
18 The human rhodopsin hybrid cDNA with a single base
19 alteration, a C-->G change (at position 477) was
20 introduced into human rhodopsin cDNA, using a HindIII
21 to BstEII PCR cassette, by primer directed PCR
22 mutagenesis. This sequence change occurs at a silent
23 position - it does not give rise to an amino acid
24 substitution - however it eliminates the ribozyme
25 cleavage site (GUX -->GUG). The hybrid rhodopsin was
26 cloned into pCDNA3 in a 5' to 3' orientation allowing
27 subsequent expression of RNA from the T7 or CMV
28 promoter in the vector (Sequence 2).

29

30 Rhodopsin cDNA carrying a Pro23Leu adRP mutation
31 A human rhodopsin adRP mutation, a C-->T change (at
32 codon 23) was introduced into human rhodopsin cDNA,
33 using a HindIII to BstEII PCR cassette by primer
34 directed PCR mutagenesis. This sequence change results
35 in the substitution of a Proline for a Leucine residue.
36 Additionally the nucleotide change creates a ribozyme

1 cleavage site (CCC-->CTC). The mutated rhodopsin was
2 cloned into the HindIII and EcoRI sites of pCDNA3 in a
3 5' to 3' orientation allowing subsequent expression of
4 RNA from the T7 or CMV promoter in the vector
5 (Sequence 3).

6
7 **Ribozyme constructs**

8 A hammerhead ribozyme (termed Rz10) designed to target
9 a large conserved open loop structure in the RNA from
10 the coding regions of the gene was cloned subsequent to
11 synthesis and annealing into the HindIII and XbaI sites
12 of pCDNA3 again allowing expression of RNA from the T7
13 or CMV promoter in the vector (Sequence 4). The target
14 site was GUC (the GUX rule) at position 475-477 of the
15 human rhodopsin sequence. A hammerhead ribozyme
16 (termed Rz20) designed to target an open loop structure
17 in RNA from the coding region of a mutant rhodopsin
18 gene with a Pro23Leu mutation was cloned subsequent to
19 synthesis and annealing into the HindIII and XbaI sites
20 of pCDNA3 again allowing expression of RNA from the T7
21 or CMV promoter in the vector (Sequence 5). The target
22 site was CTC (the NUX rule) at codon 23 of the human
23 rhodopsin sequence (Accession number: K02281).

24 Antisense flanks are underlined.

25 Rz10: GGACGGTCTGATGAGTCCGTGAGGACGAAACGTAGAG

26 Rz20: TACTCGAACTGATGAGTCCGTGAGGACGAAAGGCTGC

27
28 **Mouse rhodopsin**

29
30 **Template cDNA**

31 The full length mouse rhodopsin cDNA was cloned into
32 the EcoRI sites of the MCS of pCDNA3 in a 5' to 3'
33 orientation allowing subsequent expression of RNA from
34 the T7 or CMV promoter in the vector (Sequence 6).

35
36 cDNA with altered sequence at a wobble position

1 The mouse rhodopsin hybrid cDNA with a single base
2 alteration, a T-->C change (at position 1460) was
3 introduced into mouse rhodopsin cDNA, using a HindIII
4 to Eco47III PCR cassette, by primer directed PCR
5 mutagenesis. This sequence change occurs at a silent
6 position - it does not give rise to an amino acid
7 substitution - however it eliminates the ribozyme
8 cleavage site (TTT-->TCT). The hybrid rhodopsin was
9 cloned into pCDNA3 in a 5' to 3' orientation allowing
10 subsequent expression of RNA from the T7 or CMV
11 promoter in the vector (Sequence 7).
12

13 Ribozyme constructs

14 A hammerhead ribozyme (termed Rz33) designed to target
15 a large robust open loop structure in the RNA from the
16 coding regions of the gene was cloned subsequent
17 to synthesis and annealing into the HindIII and XbaI
18 sites of pCDNA3 again allowing expression of RNA from
19 the T7 or CMV promoter in the vector (Sequence
20 8). The target site was TTT (the NUX rule) at position
21 1459-1461 of the mouse rhodopsin sequence. (Accession
22 number: M55171). Antisense flanks are underlined.
23 Rz33: GGCACATCTGATGAGTCCGTGAGGACGAAAAAATTGG
24

25 Human peripherin

26

27 Template cDNA

28 The full length human peripherin cDNA was cloned into
29 the EcoRI sites of the MCS of pCDNA3 in a 5' to 3'
30 orientation allowing subsequent expression of RNA from
31 the T7 or CMV promoter in the vector (Sequence 9).
32

33 DNAs with altered sequence at a wobble position

34 A human peripherin hybrid DNA with a single base
35 alteration, a A-->G change (at position 257) was
36 introduced into human peripherin DNA by primer directed

1 PCR mutagenesis (forward 257 mutation primer -
2 5'CATGGCGCTGCTGAAAGTCA3'- the reverse 257 primer was
3 complementary to the forward primer). This sequence
4 change occurs at a silent position - it does not give
5 rise to an amino acid substitution - however it
6 eliminates the ribozyme cleavage site
7 (CTA-->CTG) (Sequence 10). A second human peripherin
8 hybrid DNA with a single base alteration, a A-->G
9 change (at position 359) was introduced into human
10 peripherin DNA, again by primer directed PCR
11 mutagenesis (forward 359 mutation primer -
12 5'CATCTTCAGCCTGGGACTGT3' - the reverse 359 primer was
13 complementary to the forward primer) (Sequence 11).
14 Similarly this sequence change occurs at a silent
15 position - it does not give rise to an amino acid
16 substitution - however again it eliminates the ribozyme
17 cleavage site (CTA-->CTG). The ribozyme cleavage sites
18 at 255-257 and 357-359 occur at different open loop
19 structures as predicted by the RNAPlotFold program.
20 Hybrid peripherin DNAs included the T7 promoter
21 sequence allowing subsequent expression of RNA.

22

23 Ribozyme constructs

24 Hammerhead ribozymes (termed Rz30 and Rz31), designed
25 to target robust open loop structures in the RNA from
26 the coding regions of the gene, were cloned subsequent
27 to synthesis and annealing into the HindIII and XbaI
28 sites of pCDNA3 again allowing expression of RNA from
29 the T7 or CMV promoter in the vector (Sequences
30 12+13). The target sites were both CTA (the NUX rule)
31 at positions 255-257 and 357-359 respectively of the
32 human peripherin sequence. (Accession number: M73531).
33 Antisense flanks are underlined.

34 Rz30: ACTTTCAGCTGATGAGTCCGTGAGGACGAAAGCGCCA

35 Rz31: ACAGTCCCTGATGAGTCCGTGAGGACGAAAGGCTGAA

36

1 Human Type I Collagen - COL1A2

2

3 Template cDNA

4 A human type I collagen 1A2 cDNA was obtained from the
5 ATCC (Accession No: Y00724). A naturally occurring
6 polymorphism has previously been found in collagen 1A2
7 at positions 907 of the gene involving a T-->A
8 nucleotide change (Filie et al. 1993). The
9 polymorphism occurs in a predicted open loop structure
10 of human collagen 1A2 RNA. Polymorphic variants of
11 human collagen 1A2 were generated by PCR directed
12 mutagenesis, using a HindIII to XbaI PCR cassette.
13 Resulting clones contained the following polymorphism :
14 collagen 1A2 (A) has a A nucleotide at position 907
15 (Sequence 14). In contrast human collagen 1A2 (B) has
16 a T nucleotide at position 907 (Sequence 15). In
17 collagen 1A2 (B) there is a ribozyme target site, that
18 is a GTC site (906-908), however this cleavage site is
19 lost in collagen 1A2 (A) as the sequence is altered to
20 GAC (906-908), thereby disrupting the ribozyme target
21 site.

22

23 Ribozyme constructs

24 A hammerhead ribozyme (termed Rz907) was designed to
25 target a predicted open loop structure in the RNA from
26 the coding region of the polymorphic variant of the
27 human collagen 1A2 gene. Rz907 oligonucleotide primers
28 were synthesised, annealed and cloned into the HindIII
29 and XbaI sites of pCDNA3 again allowing subsequent
30 expression of RNA from the T7 or CMV promoter in the
31 vector (Sequence 16). The target site was a GUX site
32 at position 906-908 of the human type I collagen 1A2
33 sequence (Accession number: Y00724). Antisense flanks
34 are underlined.

35 Rz907: CGGCGGCTGATGAGTCCGTGAGGACGAAACCAGCA

36

1 FIGURE LEGENDS

2

3 Diagram 1

4 pBR322 was cut with MspI, radioactively labeled and run
5 on a polyacrylamide gel to enable separation of the
6 resulting DNA fragments. The sizes of these fragments
7 are given in diagram 1. This DNA ladder was then used
8 on subsequent polyacrylamide gels (4-8%) to provide an
9 estimate of the size of the RNA products run on the
10 gels. However there is a significant difference in
11 mobility between DNA and RNA depending on the
12 percentage of polyacrylamide and the gel running
13 conditions - hence the marker provides an estimate of
14 size of transcripts.

15

16 Figure 1 -

17 A: Human rhodopsin cDNA was expressed from the T7
18 promoter to the BstEII site in the coding sequence.
19 Resulting RNA was mixed with Rz10RNA in 15mM magnesium
20 chloride and incubated at 37°C for varying times.
21 Lanes 1-4: Human rhodopsin RNA and Rz10RNA after
22 incubation at 37°C with 15mM magnesium chloride for 0,
23 1 2 and 3 hours respectively. Sizes of the expressed
24 RNAs and cleavage products are as expected (Table 1).
25 Complete cleavage of human rhodopsin RNA was obtained
26 with a small residual amount of intact RNA present at 1
27 hour. Lane 6 is intact unadapted human rhodopsin RNA
28 (BstEII) alone. Lane 5 is unadapted human rhodopsin
29 RNA (FspI) alone and refers to Figure 1B. From top to
30 bottom, human rhodopsin RNA and the two cleavage
31 products from this RNA are highlighted with arrows.
32 B: The unadapted human rhodopsin cDNA was expressed
33 from the T7 promoter to the FspI site in the coding
34 sequence. The adapted human rhodopsin cDNA was
35 expressed from the T7 promoter to the BstEII site in
36 the coding sequence. Lanes 1-4: Resulting RNAs were

1 mixed together with Rz10 and 15mM magnesium chloride
2 and incubated at 37°C for varying times (0, 1, 2 and 3
3 hours respectively). The smaller unadapted rhodopsin
4 transcripts were cleaved by Rz10 while the larger
5 adapted transcripts were protected from cleavage by
6 Rz10. Cleavage of adapted protected transcripts would
7 have resulted in products of 564bases and 287bases -
8 the 564bases product clearly is not present - the 287bp
9 product is also generated by cleavage of the unadapted
10 human rhodopsin transcripts and hence is present (FspI).
11 After 3 hours the majority of the unadapted rhodopsin
12 transcripts has been cleaved by Rz10. Lane 5 contains
13 the intact adapted human rhodopsin RNA (BstEII) alone.
14 From top to bottom adapted uncleaved human rhodopsin
15 transcripts, residual unadapted uncleaved human
16 rhodopsin transcripts and the larger of the cleavage
17 products from unadapted human rhodopsin transcripts are
18 highlighted by arrows. The smaller 22bases cleavage
19 product from the unadapted human rhodopsin transcripts
20 has run off the gel.

21

22 Figure 2

23 A: Unadapted and adapted human rhodopsin cDNAs were
24 expressed from the T7 promoter to the AcyI after the
25 coding sequence and the BstEII site in the coding
26 sequence respectively. Sizes of expressed RNAs and
27 cleavage products were as predicted (Table 1).
28 Resulting RNAs were mixed together with Rz10RNA at
29 varying magnesium chloride concentrations and incubated
30 at 37°C for 3 hours. Lane 1: Intact unadapted human
31 rhodopsin RNA (AcyI) alone. Lanes 2-5: Unadapted and
32 adapted human rhodopsin RNAs and Rz10RNA after
33 incubation at 37°C with 0, 5, 10 and 15 mM MgCl₂,
34 respectively. Almost complete cleavage of the larger
35 unadapted human rhodopsin RNA was obtained with a small
36 residual amount of intact RNA present at 5 mM MgCl₂. In

1 contrast the adapted human rhodopsin RNA remained
2 intact. From top to bottom, the unadapted and adapted
3 rhodopsin RNAs, and two cleavage products from the
4 unadapted human rhodopsin RNA are highlighted by
5 arrows. Lane 6 is intact adapted human rhodopsin RNA
6 (BstEII) alone.

7 B: The adapted human rhodopsin cDNA was expressed from
8 the T7 promoter to the BstEII site in the coding
9 sequence. Lanes 1-4: Resulting RNA was mixed together
10 with Rz10 and 0, 5, 10 and 15 mM magnesium chloride
11 and incubated at 37°C for 3 hours respectively. The
12 adapted rhodopsin transcripts were not cleaved by Rz10.
13 Cleavage of adapted transcripts would have resulted in
14 cleavage products of 564bases and 287bases which
15 clearly are not present. Lane 5: intact adapted human
16 rhodopsin RNA (BstEII) alone. Lane 4: RNA is absent -
17 due to a loading error or degradation. The adapted
18 uncleaved human rhodopsin RNA is highlighted by an
19 arrow.

20 C: Unadapted and adapted human rhodopsin cDNAs were
21 expressed from the T7 promoter to the AcyI after the
22 coding sequence and the BstEII site in the coding
23 sequence respectively. Sizes of expressed RNAs and
24 cleavage products were as predicted (Table 1).
25 Resulting RNAs were mixed together with Rz10RNA at
26 varying magnesium chloride concentrations and
27 incubated at 37°C for 3 hours. Lane 1: DNA ladder as
28 in Diagram 1. Lanes 2-5: Unadapted and adapted human
29 rhodopsin RNAs and Rz10RNA after incubation at 37°C
30 with 0, 5, 10 and 15 mM MgCl₂ respectively. Almost
31 complete cleavage of the larger unadapted human
32 rhodopsin RNA was obtained with a small residual amount
33 of intact RNA present at 5 and 10 mM MgCl₂. In contrast
34 the adapted human rhodopsin RNA remained intact. Lane
35 6: Adapted human rhodopsin RNA (BstEII) alone. Lane 7:
36 Unadapted human rhodopsin RNA (AcyI) alone. Lane 8:

1 DNA ladder as in Diagram 1. From top to bottom, the
2 unadapted and adapted rhodopsin RNAs, and two cleavage
3 products from the unadapted human rhodopsin RNA are
4 highlighted by arrows. Separation of the adapted human
5 rhodopsin RNA (851bases) and the larger of the cleavage
6 products from the unadapted RNA (896bases) is
7 incomplete in this gel (further running of the gel
8 would be required to achieve separation) - however the
9 separation of these two RNAs is demonstrated in Figure
10 2A.

11

12 Figure 3

13 The mutant (Pro23Leu) human rhodopsin cDNA was
14 expressed from the T7 promoter to the BstEII in the
15 coding sequence. Likewise the Rz20 clone was expressed
16 to the XbaI site. Resulting RNAs were mixed together
17 with 10mM magnesium chloride concentrations at 37°C for
18 varying times. Sizes of expressed RNAs and cleavage
19 products were as predicted (Table 1). Lane 1: DNA
20 ladder as in Diagram 1. Lanes 2: Pro23Leu human
21 rhodopsin RNA alone. Lanes 3-7 Pro23Leu human
22 rhodopsin RNA and Rz20 RNA after incubation at 37°C with
23 10 mM MgCl₂ for 0 mins, 30 mins, 1 hr, 2hrs and 5hrs
24 respectively. Almost complete cleavage of mutant
25 rhodopsin transcripts was obtained with a residual
26 amount of intact RNA left even after 5 hours. Lane
27 8: DNA ladder as in Diagram 1. From top to bottom,
28 intact mutant rhodopsin RNA and the two cleavage
29 products from the mutant human rhodopsin RNA are
30 highlighted by arrows.

31

32 Figure 4

33 The mutant (Pro23Leu) human rhodopsin cDNA was
34 expressed from the T7 promoter to the BstEII in the
35 coding sequence. Likewise the Rz10 clone was expressed
36 to the XbaI site. Resulting RNAs were mixed together

1 with 10mM magnesium chloride concentrations at 37°C for
2 varying times. Sizes of expressed RNAs and cleavage
3 products were as predicted (Table 1). Lane 1: DNA
4 ladder as in Diagram 1. Lanes 2: Pro23Leu human
5 rhodopsin RNA alone. Lanes 3-7 Pro23Leu human
6 rhodopsin RNA and Rz10RNA after incubation at 37°C with
7 10 mM MgCl₂ for 0mins, 30 mins, 1 hr, 2hrs and 5hrs
8 respectively. Almost complete cleavage of mutant human
9 rhodopsin RNA was obtained with a residual amount of
10 intact RNA remaining even after 5 hours (Lane 7). Lane
11 8: DNA ladder as in Diagram 1. From top to bottom,
12 intact mutant rhodopsin RNA and the two cleavage
13 products from the mutant human rhodopsin RNA
14 are highlighted by arrows.

15

16 Figure 5 -

17 The mouse rhodopsin cDNA clone was expressed in vitro
18 from the T7 promoter to the Eco47III site in the coding
19 sequence. Likewise the Rz33 clone was expressed to the
20 XbaI site. A: Resulting RNAs were mixed together with
21 10mM magnesium chloride at 37°C for varying times.
22 Sizes of expressed RNAs and cleavage products were as
23 predicted (Table 1). DNA ladder as in Diagram 1. Lane
24 1: mouse rhodopsin RNA alone. Lanes 2-5 Mouse
25 rhodopsin RNA and Rz33 RNA after incubation at 37°C
26 with 10 mM MgCl₂ at 0, 5, 7.5 and 10 mM MgCl₂
27 respectively for 3 hours. Cleavage of mouse rhodopsin
28 RNA was obtained after addition of divalent ions (Lane
29 3). Residual uncleaved mouse rhodopsin RNA and the two
30 cleavage products from the mouse rhodopsin RNA are
31 highlighted by arrows. B: The adapted mouse rhodopsin
32 cDNA clone with a base change at position 1460 was
33 expressed in vitro from the T7 promoter to the Eco47III
34 site in the coding sequence. Likewise the Rz33 clone
35 was expressed to the XbaI site. Resulting RNAs were
36 mixed together with various magnesium chloride

1 concentrations at 37°C for 3 hours. Sizes of expressed
2 RNAs and cleavage products were as predicted (Table 1).
3 Lane 1: DNA ladder as in Diagram 1. Lane 2: Adapted
4 mouse rhodopsin RNA alone. Lanes 3-6: Adapted mouse
5 rhodopsin RNA and Rz33 RNA after incubation at 37°C
6 with 0, 5, 7.5 and 10 mM MgCl for 3 hours at 37°C. No
7 cleavage of the adapted mouse rhodopsin RNA was
8 observed. The intact adapted mouse rhodopsin RNA is
9 highlighted by an arrow.

10

11 Figure 6

12 The human peripherin cDNA clone was expressed *in vitro*
13 from the T7 promoter to the BglII site in the coding
14 sequence. Likewise Rz30 (targeting a cleavage site
15 at 255-257) was expressed to the XbaI site. A:
16 Resulting RNAs were mixed together with 10mM magnesium
17 chloride at 37°C for varying times. Lane 1: DNA ladder
18 as in Diagram 1. Lane 2: Intact human peripherin RNA
19 alone. Lanes 3-7: Human peripherin RNA and Rz30 RNA
20 after incubation at 37°C with 10 mM MgCl for 3hrs,
21 2hrs, 1hr, 30 mins and 0 mins respectively. Lane 8:
22 DNA ladder as in Diagram 1. From top to bottom, intact
23 human peripherin RNA and the two cleavage products from
24 the human peripherin RNA are highlighted by arrows. B:
25 Resulting RNAs were mixed with Rz30 RNA at varying
26 magnesium chloride concentrations and incubated at 37°C
27 for 3hrs. Lane 1: DNA ladder as in Diagram 1. Lanes
28 2-5: Human peripherin RNA and Rz30 after incubation at
29 37°C with 10, 7.5, 5 and 0 mM magnesium chloride
30 respectively for 3hrs. Lane 6: Intact human peripherin
31 RNA alone. Sizes of the expressed RNAs and cleavage
32 products are as expected (Table 1). Significant
33 cleavage of human peripherin RNA was obtained with a
34 residual amount of intact RNA present at each MgCl₂
35 concentration. From top to bottom, human peripherin
36 RNA and the two cleavage products from this RNA are

1 highlighted with arrows. C: The adapted human
2 peripherin DNA with a single base change at position
3 257 was expressed from the T7 promoter to the AvrII
4 site in the coding sequence. Resulting RNA was mixed
5 with Rz30 at various magnesium chloride concentrations
6 and incubated at 37°C for 3hrs. Lane 1: DNA ladder as
7 in Diagram 1. Lane 2: Intact adapted human peripherin
8 RNA alone. Lanes 3-6: Adapted human peripherin RNA and
9 Rz30 after incubation at 37°C with 0, 5, 7.5 and 10 mM
10 magnesium chloride respectively for 3hrs. Lane 7: DNA
11 ladder as in Diagram 1. D: The unadapted human
12 peripherin cDNA and the adapted human peripherin DNA
13 fragment with a single base change at position 257 were
14 expressed from the T7 promoter to the BglII and AvrII
15 sites respectively in the coding sequence. Resulting
16 RNAs were mixed with Rz30 at various magnesium chloride
17 concentrations and incubated at 37°C for 3hrs. Lane 1:
18 DNA ladder as in Diagram 1. Lane 2: Intact unadapted
19 human peripherin RNA alone. Lane 3: Intact adapted
20 human peripherin RNA alone. Lanes 4-7: Unadapted and
21 adapted human peripherin RNAs and Rz30 after incubation
22 at 37°C with 0, 5, 7.5 and 10 mM magnesium chloride
23 respectively for 3hrs at 37°C. No cleavage of the
24 adapted human peripherin RNA was observed even after 3
25 hours whereas a significant reduction in the unadapted
26 RNA was observed over the same time frame. Lane 8: DNA
27 ladder as in Diagram 1. From top to bottom, residual
28 unadapted human peripherin RNA, adapted human
29 peripherin RNA and the two cleavage products are
30 highlighted by arrows.

31

32 Figure 7

33 Human peripherin cDNA clone was expressed in vitro from
34 the T7 promoter to the BglII site in the coding
35 sequence. Likewise the Rz31 (targeting a cleavage site
36 at 357-359) was expressed to the XbaI site. A:

1 Resulting RNAs were mixed together with 10mM magnesium
2 chloride at 37°C for varying times. Lane 1: DNA ladder
3 as in Diagram 1. Lanes 2-6: Human peripherin RNA and
4 Rz31 RNA after incubation at 37°C with 10mM MgCl for
5 3hrs, 2hrs, 1hr, 30mins and 0mins respectively.
6 Increased cleavage of mouse rhodopsin RNA was obtained
7 over time - however significant residual intact RNA
8 remained even after 3 hours (Lane 2). Lane 7: Intact
9 human peripherin RNA alone. Lane 8: DNA ladder as in
10 Diagram 1. From top to bottom, intact human peripherin
11 RNA and the two cleavage products from the human
12 peripherin RNA are highlighted by arrows. B: Resulting
13 RNAs were mixed with Rz31 RNA at varying magnesium
14 chloride concentrations and incubated at 37°C for 3hrs.
15 Lane 1: DNA ladder as in Diagram 1. Lanes 2-5: Human
16 peripherin RNA and Rz31 after incubation at 37°C with
17 10, 7.5, 5 and 0m M magnesium chloride respectively for
18 3hrs. Sizes of the expressed RNAs and cleavage
19 products are as expected (Table 1). Significant
20 cleavage of human peripherin RNA was obtained with a
21 residual amount of intact RNA present at each MgCl₂
22 concentration (Lanes 2-4). Lane 6: Intact human
23 peripherin RNA alone. Lane 7: DNA ladder as in Diagram
24 1. From top to bottom, human peripherin RNA and the
25 two cleavage products from this RNA are highlighted
26 with arrows. C: The adapted human peripherin DNA with
27 a single base change at position 359 was expressed from
28 the T7 promoter to the BglII site in the coding
29 sequence. Resulting RNA was mixed with Rz31 at various
30 magnesium chloride concentrations and incubated at 37°C
31 for 3hrs. Lane 1: DNA ladder as in Diagram 1. Lane 2:
32 Intact adapted human peripherin RNA alone. Lanes 3-6:
33 Adapted human peripherin RNA and Rz31 after incubation
34 at 37°C with 0, 5, 7.5 and 10mM magnesium chloride
35 respectively for 3hrs. No cleavage of the adapted
36 human peripherin RNA was observed even after 3 hours.

1 Lane 7: DNA ladder as in Diagram 1.

2

3 Figure 8

4 A: The human collagen 1A2 cDNA clones containing the A
5 and T alleles of the polymorphism at position 907 were
6 expressed from the T7 promoter to the MvnI and XbaI
7 sites in the insert and vector respectively. Resulting
8 RNAs were mixed together with Rz907 and various MgCl₂
9 concentrations and incubated at 37°C for 3 hours. Lane
10 1: intact RNA from the human collagen 1A2 (A)
11 containing the A allele of the 907 polymorphism. Lane
12 2: intact RNA from the human collagen 1A2 (B)
13 containing the T allele of the 907 polymorphism. Lanes
14 3-5: Human collagen 1A2 (A) and (B) representing the A
15 and T allele RNAs and Rz907 incubated with 0, 5, and 10
16 mM MgCl₂ at 37°C for 3 hours. RNA transcripts from the
17 T allele containing the 906-908 target site are cleaved
18 by Rz907 upon addition of divalent ions - almost
19 complete cleavage is obtained with a residual amount of
20 transcript from the T allele remaining (Lane 5). In
21 contrast transcripts expressed from the A allele (which
22 are smaller in size to distinguish between the A (MvnI)
23 and T (XbaI) alleles) were not cleaved by Rz907 - no
24 cleavage products were observed. From top to bottom,
25 RNA from the T allele, the A allele and the two
26 cleavage products from the T allele are highlighted by
27 arrows. Lane 6: DNA ladder as in Diagram 1.

28 B: The human collagen 1A2 cDNA (A) + (B) clones
29 containing the A and T alleles of the polymorphism at
30 907 were expressed from the T7 promoter to the MvnI and
31 XbaI sites in the insert and vector respectively.
32 Resulting RNAs were mixed together with Rz907 and 10mM
33 magnesium chloride and incubated at 37°C for varying
34 times. Lane 1: DNA ladder as in Diagram 1. Lane 2:
35 intact RNA from the human collagen 1A2 (A) with the A
36 allele of the 907 polymorphism. Lane 3: intact RNA

1 from the human collagen 1A2 (B) with the T allele of
2 the 907 polymorphism. Lanes 4-9: Human collagen 1A2 A
3 and T allele RNA and Rz907 incubated with 10mM MgCl₂ at
4 37°C for 0, 30 mins, 1hour, 2hours, 3 hours and
5 5hours respectively. RNA transcripts from the T allele
6 containing the 906-908 target site are cleaved by Rz907
7 - almost complete cleavage is obtained after 5 hours.
8 In contrast transcripts expressed from the A allele
9 (which are smaller in size to distinguish between the A
10 (MvnI) and T (XbaI) alleles) were not cleaved by Rz907
11 - no cleavage products were observed. From top to
12 bottom, RNA from the T allele, the A allele and the two
13 cleavage products from the T allele are highlighted by
14 arrows.

1 Sequences

2

3 Sequence 1

4 The human rhodopsin cDNA in pCDNA3.

5

6 Sequence 2

7 The human rhodopsin cDNA in pCDNA3 with a base change
8 at a silent site (477).

9

10 Sequence 3

11 Mutant (Pro23Leu) human rhodopsin cDNA in pCDNA3.

12

13 Sequence 4

14 Rz10 cloned into pCDNA3. Note there is a one base
15 mismatch in one antisense arm of Rz10.

16

17 Sequence 5

18 Rz20 cloned into pCDNA3

19

20 Sequence 6

21 The mouse rhodopsin cDNA in pCDNA3.

22

23 Sequence 7

24 The mouse rhodopsin cDNA in pCDNA3 with a base change
25 at a silent site (1460).

26

27 Sequence 8

28 Rz33 cloned into pCDNA3

29

30 Sequence 9

31 The human peripherin cDNA in pCDNA3.

32

33 Sequence 10

34 The human peripherin DNA fragment with a base change at
35 a silent site (257).

36

- 1 Sequence 11
- 2 The human peripherin DNA fragment with a base change at
- 3 a silent site (359).
- 4
- 5 Sequence 12
- 6 Rz30 cloned into pCDNA3
- 7
- 8 Sequence 13
- 9 Rz31 cloned into pCDNA3
- 10
- 11 Sequence 14
- 12 Collagen 1A2 (A) sequence containing the A polymorphism
- 13 at position 907. (Note there is an additional
- 14 polymorphic site at position 902).
- 15
- 16 Sequence 15
- 17 Collagen 1A2 (B) sequence containing the T polymorphism
- 18 at position 907. (Note there is an additional
- 19 polymorphic site at position 902).
- 20
- 21 Sequence 16
- 22 Rz907 cloned into pCDNA3
- 23
- 24 **Note:**
- 25
- 26 The sequence quality was not good in the region of the
- 27 human peripherin 359 silent change - the sequencing
- 28 primer was too far from the target site to achieve good
- 29 quality sequence (Sequence 11).

1 RESULTS

2

3 Human and mouse rhodopsin, human peripherin and human
4 collagen 1A2 cDNA clones were expressed *in vitro*.
5 Ribozymes targeting specific sites in the human and
6 mouse rhodopsin, human peipherin and human collagen 1A2
7 cDNAs were also expressed *in vitro*. cDNA clones were
8 cut with various restriction enzymes resulting in the
9 production of differently sized transcripts after
10 expression. This aided in differentiating between RNAs
11 expressed from unadapted and adapted cDNAs.
12 Restriction enzymes used to cut each clone, sizes of
13 resulting transcripts and predicted sizes of products
14 after cleavage by target ribozymes are given below in
15 Table 1. Exact sizes of expression products may vary
16 by a few bases from that estimated as there may be some
17 ambiguity concerning inter alia the specific base at
18 which transcription starts.

19

20 Example 1

21

22 A: Human Rhodopsin

23

24 The unadapted human rhodopsin cDNA and the human
25 rhodopsin cDNA with a single nucleotide substitution in
26 the coding sequence were cut with BstEII and expressed
27 *in vitro*. The single base change occurs at the third
28 base position or wobble position of the codon (at
29 position 477) and therefore does not alter the amino
30 acid coded by this triplet. The Rz10 clone was cut
31 with XbaI and expressed *in vitro*. Resulting ribozyme
32 and human rhodopsin RNAs were mixed with varying
33 concentrations of MgCl₂ to optimise cleavage of template
34 RNA by Rz10. A profile of human rhodopsin RNA cleavage
35 by Rz10 over time is given in Figure 1A. The
36 MgCl₂ curve profile used to test if adapted human

1 rhodopsin transcripts could be cleaved by Rz10 is given
2 in Figure 2B. Unadapted and adapted human rhodopsin
3 cDNAs were cut with FspI and BstEII respectively,
4 expressed and mixed together with Rz10 RNA to test for
5 cleavage (Figure 1B) over time. Likewise, unadapted
6 and adapted human rhodopsin cDNAs were cut with AcyI
7 and BstEII respectively, both were expressed in vitro
8 and resulting transcripts mixed with Rz10 RNA at
9 varying $MgCl_2$ concentrations to test for cleavage
10 (Figure 2A, 2C). In all cases expressed RNAs were the
11 predicted size. Similarly in all cases unadapted
12 transcripts were cleaved into products of the predicted
13 size. Cleavage of unadapted human rhodopsin RNA was
14 almost complete - little residual uncleaved RNA
15 remained. In all cases adapted human rhodopsin RNAs
16 with a single base change at a silent site remained
17 intact, that is, it was not cleaved by Rz10. Clearly,
18 transcripts from the unadapted human rhodopsin cDNA are
19 cleaved by Rz10 while transcripts from the adapted
20 replacement gene which has been modified in a manner
21 which exploits the degeneracy of the genetic code are
22 protected from cleavage. It is worth noting that AcyI
23 enzyme cuts after the stop codon and therefore the
24 resulting RNA includes the complete coding sequence of
25 the gene.

26

27 B: Human Rhodopsin

28

29 Rz20 was cut with XbaI and expressed in vitro.
30 Similarly the rhodopsin cDNA containing a Pro23Leu
31 mutation was cut with BstEII and expressed in vitro.
32 Resulting RNAs were mixed and incubated at 37°C with
33 10mM $MgCl_2$ for varying times. Rz20 was designed to
34 elicit mutation specific cleavage of transcripts
35 containing a Pro23Leu rhodopsin mutation. All
36 expressed products and cleavage products were the

1 correct size. Figure 3 demonstrates mutation specific
2 cleavage of the mutant RNA over time incubated at 37°C
3 with 10mM MgCl₂. Cleavage of mutant rhodopsin
4 transcripts by Rz10 which targets a ribozyme cleavage
5 site 3' of the site of the Pro23Leu mutation in
6 rhodopsin coding sequence was explored. The mutant
7 rhodopsin cDNA and Rz10 clones were cut with BstEII and
8 XbaI respectively and expressed in vitro. Resulting
9 RNAs were mixed and incubated with 10mM MgCl₂ for
10 varying times (Figure 4). All expressed products and
11 cleavage products were the correct size. Rz10 cleaved
12 mutant rhodopsin transcripts. Using a replacement gene
13 with a sequence change around the Rz10 cleavage site
14 which is at a wobble position we demonstrated in
15 Example 1A that transcripts from the replacement gene
16 remain intact due to absence of the Rz10 target site
17 (Figures 1B, 2A and 2B). Hence Rz10 could be used to
18 cleave mutant transcripts in a manner independent of
19 the disease mutation itself (that is, using this site)
20 while transcripts from the replacement gene which code
21 for the correct protein would remain intact and
22 therefore could supply the wild type protein.

23

24 Example 2

25 Mouse Rhodopsin

26 Rz33 was cut with XbaI and expressed in vitro.
27 Similarly the mouse rhodopsin cDNA was cut with
28 Eco47III and expressed in vitro. Resulting RNAs were
29 mixed and incubated with varying concentrations of
30 MgCl₂. All expressed products and cleavage products
31 were the correct size. Figure 5A demonstrates specific
32 cleavage of the mouse rhodopsin RNA over various MgCl₂
33 concentrations incubated at 37°C for 3 hours. Using a
34 replacement gene with a sequence change around the Rz33
35 cleavage site (TTT-->TCT) which is at a wobble position
36 we demonstrated that transcripts from the replacement

1 gene remain intact due to absence of the Rz33 target
2 site (Figures 5B). Hence Rz33 could be used to cleave
3 mutant transcripts in a manner independent of the
4 disease mutation itself (that is, using this site)
5 while transcripts from the replacement gene which code
6 for the correct protein would remain intact and
7 therefore could supply the wild type protein.

8

9 Example 3

10 Human Peripherin

11 The unadapted human peripherin cDNA and two human
12 peripherin DNA fragments generated by PCR mutagenesis
13 with a single nucleotide substitution in the coding
14 sequence were cut with BglII and AvrII respectively and
15 expressed in vitro. The single base changes in the
16 adapted DNAs occur at third base positions or wobble
17 positions of the codon (at position 257 and 359) and
18 therefore do not alter the amino acid coded by these
19 triplets. The Rz30 and Rz31 clones were cut with XbaI
20 and expressed in vitro. Resulting ribozymes and
21 unadapted human rhodopsin RNAs were mixed with varying
22 concentrations of MgCl₂ to optimise cleavage of template
23 RNA by Rz30 and Rz31. Profiles of human peripherin RNA
24 cleavage by Rz30 over various MgCl₂ concentrations and
25 over time are given in Figure 6. Similarly profiles of
26 human peripherin RNA cleavage by Rz31 over various MgCl₂
27 concentrations and over time are given in Figure 7. In
28 all cases expressed RNAs were the predicted size.
29 Similarly in all cases unadapted transcripts were
30 cleaved into products of the predicted size. Adapted
31 human rhodopsin RNAs were mixed together with Rz30 and
32 Rz31 RNA over various MgCl₂ concentrations to test if
33 adapted human peripherin transcripts could be cleaved
34 by Rz30 and Rz31 (Figures 6 + 7). Expressed RNAs were
35 the predicted size. In all cases adapted human
36 peripherin RNAs with single base changes at silent

1 sites remained intact, that is, they were not cleaved
2 by Rz30 or Rz31. Clearly, transcripts from the
3 unadapted human peripherin cDNA are cleaved by Rz30 and
4 Rz31 while transcripts from the adapted replacement
5 DNAs which have been modified in a manner which
6 exploits the degeneracy of the genetic code are
7 protected from cleavage.

8

9 Example 4

10 Human Collagen 1A2

11 Rz907 clones targeting a polymorphic site in human
12 collagen 1A2 sequence was cut with XbaI and expressed
13 *in vitro*. The human collagen 1A2 cDNA clones (A and B)
14 containing two allelic forms of a polymorphism in the
15 coding sequence of the gene at positions 907 were cut
16 with MvnI and XbaI respectively, expressed *in vitro* and
17 RNAs mixed together with Rz907 RNA to test for cleavage
18 of transcripts by this ribozyme. All expressed
19 transcripts were of the predicted sizes. RNAs were
20 mixed with varying concentrations of MgCl₂ to optimise
21 cleavage of RNAs by Rz907 (Figure 8). Notably the
22 majority of the RNA transcripts from human collagen 1A2
23 (B) which has a T nucleotide at position 907 is cleaved
24 by Rz907 (Figure 8). This allelic form of the gene has
25 a ribozyme cleavage site at 906-908. Notably the
26 situation is reversed with transcripts from human
27 collagen 1A2 (A) where in this allelic form of the gene
28 due to the nature of the polymorphism present at
29 position 907 the ribozyme cleavage site has been lost.
30 In contrast to transcripts from human collagen (B),
31 transcripts from human collagen (A) were protected from
32 cleavage by Rz907 due to the alteration in the sequence
33 around the ribozyme cleavage site (Figure 8). Cleavage
34 of collagen 1A2 (B) by Rz907 was efficient which is
35 consistent with 2-D predictions of RNA open loop
36 structures for the polymorphism - in the allele

1 containing the Rz907 ribozyme cleavage site, the target
2 site is found quite consistently in an open loop
3 structure. This polymorphism found in an open loop
4 structure of the transcript clearly demonstrates the
5 feasibility and utility of using the degeneracy of the
6 genetic code in the suppression of an endogenous gene
7 (either suppressing both alleles or a single allele at
8 a polymorphic site) and restoration of gene expression
9 using a gene which codes for the same protein but has
10 sequence modifications at third base wobble positions
11 which protect the replacement gene from suppression.

TABLE 1

	Restriction Enzyme	RNA Size	Cleavage Products
Example 1			
Human rhodopsin	BstEII	851 bases	287+564 bases
	AcyI	1183 bases	287+896 bases
	FspI	309 bases	287+22
Adapted Human rhodopsin	BstEII	851 bases	
Human rhodopsin Pro-Leu	BstEII	851 bases	170+681 (Rz20)
Human rhodopsin Pro-Leu	BstEII	851 bases	287+564 (Rz10)
Rz10	XbaI	52 bases	
Rz20	XbaI	52 bases	
(Table 1; Sequences 1-5 ; Figures 1-4)			
Example 2			
Mouse rhodopsin	Eco47III	774 bases	400+374
Adapted mouse rhodopsin	Eco47III	774 bases	
Rz33	XbaI	52 bases	
(Table 1; Sequences 6-9; Figure 5)			
Example 3			
Human peripherin	BglII	545 bases	315+230 (Rz30)
Human peripherin	BglII	545 bases	417+128 (Rz31)
Adapted human peripherin	AvrII	414 bases	
Adapted human peripherin	BglII	545 bases	
Rz30	XbaI	52 bases	
Rz31	XbaI	52 bases	
(Table 1; Sequences 10-14; Figures 6+7)			
Example 4			
Human Collagen 1A2 (B) -Rz907	XbaI	888 bases	690+198 bases
Human Collagen 1A2 (A)	MvnI	837 bases	
Rz907	XbaI	52 bases	
(Table 1; Sequences 14-16; Figure 8)			

TABLE 2

A: Rhodopsin mutations tested to assess if the predicted open loop RNA structure containing the Rz10 target site (475-477) remains intact in mutant transcripts.

Rhodpsin mutation	RNA open loop targeted by Rz10
Pro 23 Leu	Intact
Gly 51 Val	Intact
Thr 94 Ile	Intact
Gly 188 Arg	Intact
Met 207 Arg	Intact
Ile del 255	Intact

B: Utilisation of the degeneracy of the genetic code.
Ribozyme cleavage sites are underlined

Human rhodopsin

	475-477
Unadapted sequence	TAC <u>GTC</u> ACC GTC CAG Val
	475-477
Adapted sequence	TAC GTG ACC GTC CAG Val

Mouse rhodopsin

	1459-1461
Unadapted sequence	AAT <u>TTT</u> TAT GTG CCC Phe
	1459-1461
Adapted sequence	AAT TTC TAT GTG CCC Phe

Human peripherin

	255-257
Unadapted sequence	GCG <u>CTA</u> CTG AAA GTC Leu
	255-257
Adapted sequence	GCG CTG CTG AAA GTC Leu
	357-359
Unadapted sequence	AGC <u>CTA</u> GGA CTG TTC Leu
	357-359
Adapted sequence	AGC CTG GGA CTG TTC Leu

Human type I collagen 1A2

	906-908
Sequence (B)	GCT <u>GGT</u> CCC GCC GGT Gly

GCT GGA CCC GCC GGT
Gly

2

3 In the examples outlined above, RNA was expressed from
4 cDNAs coding for four different proteins: human and
5 mouse rhodopsin, human peripherin and human type I
6 collagen 1A2. Rhodopsin and peripherin have been used
7 to exemplify the invention for retinopathies such as
8 adRP - suppression effectors have been targeted to the
9 coding sequences of these genes. In the case of the
10 human collagen 1A2 gene a naturally occurring
11 polymorphism has been used to demonstrate the invention
12 and the potential use of the invention for disorders
13 such as OI - however non-polymorphic regions of the
14 collagen 1A2 gene could be used to achieve suppression.
15 The suppression effectors of choice in the invention
16 have been hammerhead ribozymes with antisense flanks to
17 define sequence specificity. Hammerhead ribozymes
18 require NUX cleavage sites in open loop structures of
19 RNA. Notably, other suppression effectors could be
20 utilised in the invention and may lead to a more
21 flexible choice of target sequences for suppression.
22 Transcripts expressed from all four genes have been
23 significantly attacked *in vitro* using suppression
24 effectors directed towards target cleavage sites. In
25 all four examples the ribozymes directed to cleavage
26 sites were successful in cleaving target RNAs in the
27 predicted manner. Antisense complementary to sequences
28 surrounding the cleavage sites was used successfully
29 to elicit binding and cleavage of target RNAs in a
30 sequence specific manner. Additionally, transcripts
31 from replacement genes, modified using the degeneracy
32 of the genetic code so that they code for wild type
33 protein, were protected fully from cleavage by

1 ribozymes.

2

3 The utility of an individual ribozyme designed to
4 target an NUX site in an open loop structure of
5 transcripts from a gene will depend in part on the
6 robust nature of the RNA open loop structure when
7 various deleterious mutations are also present in the
8 transcript. To evaluate this, we analysed RNAPlotFold
9 data for six different adRP causing mutations in the
10 rhodopsin gene. For each of these, the large RNA open
11 loop structure which is targeted by Rz10 was predicted
12 to be maintained in the mutant transcripts (Table 2A).
13 This is clearly demonstrated in example 1B (Figure 3)
14 using a Pro23Leu rhodopsin mutation. Rz10 clearly
15 cleaves the mutant transcript effectively in vitro.
16 The Pro23Leu mutation creates a ribozyme cleavage site
17 and can be cleaved in vitro by Rz20 a ribozyme
18 specifically targeting this site - however this is not
19 the case for many mutations. In contrast we have shown
20 that the Rz10 ribozyme cleavage site is available for
21 different mutant rhodopsins and could potentially be
22 used to suppress multiple mutations using a suppression
23 and replacement approach.

24

25 In some cases lowering RNA levels may lead to a
26 parallel lowering of protein levels however this may
27 not always be the case. In some situations mechanisms
28 may prevent a significant decrease in protein levels
29 despite a substantial decrease in levels of RNA.
30 However in many instances suppression at the RNA level
31 has been shown to be effective (see prior art). In some
32 cases it is thought that ribozymes elicit suppression
33 not only by cleavage of RNA but also by an antisense
34 effect due to the antisense arms of the ribozyme
35 surrounding the catalytic core.

36

1 In all examples provided ribozymes were designed to
2 cleave at specific target sites. Target sites for four
3 of the ribozymes utilised were chosen in open loop
4 structures in the coding regions of transcripts from
5 three retinal genes (human and mouse rhodopsin and
6 human peripherin). In all cases sequence specific
7 cleavage was obtained at the target cleavage sites
8 (Figs 1-7). Target sites were chosen in open loop
9 structures to optimise cleavage. Additionally target
10 sites were chosen such that they could be obliterated
11 by single nucleotide changes at third base wobble
12 positions and therefore would code for the same amino
13 acid (Table 2B). In turn this enabled the generation
14 of replacement genes with single nucleotide alterations
15 which code for wild type protein. In all cases tested
16 transcripts from replacement genes were protected from
17 cleavage by ribozymes. Further modifications could be
18 made to replacement genes in wobble positions, for
19 example, to limit the binding ability of the antisense
20 arms flanking the ribozyme catalytic core. The examples
21 provided for rhodopsin and peripherin involve
22 suppression of expression of both disease and wild type
23 alleles of a retinal gene and restoration of the wild
24 type protein using a replacement gene. However, there
25 may be situations where single alleles can be targeted
26 specifically or partially specifically
27 (PCT/GB97/00574).

28
29 In one example, human collagen 1A2, Rz907 was used to
30 target a naturally occurring polymorphic site at amino
31 acid 187, (GGA (glycine) --> GGT (glycine), located in
32 an open loop structure from the predicted 2-D
33 conformations of the transcript (Figure 8, Table 2B).
34 The ribozyme Rz907 cleaved transcripts containing the
35 GGT sequence but transcripts with GGA were protected
36 from cleavage. Transcripts from both alleles of

1 individuals homozygous for the GGT polymorphism could
2 be cleaved by Rz907 whereas in the case of
3 heterozygotes cleavage could be directed to single
4 alleles (in particular to alleles containing
5 deleterious mutations PCT/GB97/00574). In both
6 situations replacement genes could have the sequence
7 GGA and therefore would be protected from cleavage by
8 Rz907. The presence of many such naturally occurring
9 silent polymorphisms highlights that replacement genes
10 could be modified in a similar fashion in wobble
11 positions and should produce in most cases functional
12 wild type protein. Multiple modifications could be
13 made to replacement genes at wobble positions
14 which would augment protection from suppression
15 effectors. For example, in situations where antisense
16 nucleic acids were used for suppression, transcripts
17 from replacement genes with multiple modifications at
18 third base positions would be protected partially or
19 completely from antisense binding.

20
21 In all four examples provided transcripts from cDNA
22 clones were cleaved in vitro in a sequence specific
23 manner at ribozyme cleavage sites. Additionally one
24 base of the ribozyme cleavage site occurs at a wobble
25 position and moreover can be altered so as to eliminate
26 the cleavage site. Ribozyme cleavage sites in the
27 examples given were destroyed by changing nucleotide
28 sequences so that the consensus sequence for ribozyme
29 cleavage sites was broken. However it may be
30 that in some cases the cleavage site could be destroyed
31 by altering the nucleotide sequence in a manner that
32 alters the 2-D structure of the RNA and destroys the
33 open loop structure targeted by the ribozyme. cDNAs or
34 DNA fragments with altered sequences in the regions
35 targeted by ribozymes were generated. RNAs expressed
36 from these cDNAs or DNA fragments were protected

1 entirely from cleavage due to the absence of the
2 ribozyme cleavage site for each of the ribozymes
3 tested. Of particular interest is the fact that a
4 single nucleotide alteration can obliterate a ribozyme
5 target site, thereby preventing RNA cleavage. Although
6 ribozymes have been used in the demonstration of the
7 invention, other suppression effectors could be used to
8 achieve gene silencing. Again replacement genes with
9 altered sequences (at third base wobble positions)
10 could be generated so that they are protected partially
11 or completely from gene silencing and provide the wild
12 type (or beneficial) gene product.

13
14 As highlighted before in the text, using the invention
15 the same method of suppression (targeting coding
16 sequences of a gene) and where necessary gene
17 replacement (using a replacement gene with a sequence
18 modified in third base positions to restore gene
19 expression) may be used as a therapeutic approach for
20 many different mutations within a given gene. Given
21 the continuing elucidation of the molecular
22 pathogenesis of dominant and polygenic diseases the
23 number of targets for this invention is rapidly
24 increasing.

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- 29
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- 32
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- 34
- 35 Zhuang J et al. (1996) Hum Mut 7: 89-99.

1 **CLAIMS**

2

- 3 1. A strategy for suppressing at least a part of the
4 coding region of an endogenous gene and replacing
5 the suppressed gene sequence with a nucleic acid
6 sequence which differs from the endogenous gene.
7
- 8 2. A strategy as claimed in Claim 1 wherein the
9 suppressing agent comprises at least one
10 suppressor from the group comprising antisense
11 nucleic acid, DNA capable of forming triple helix
12 or ribozymes to the endogenous gene.
13
- 14 3. A strategy as claimed in Claim 1 or 2 wherein the
15 replacement nucleic acid sequence encodes at least
16 part of a gene product and does not interact with
17 the suppressing agent.
18
- 19 4. A strategy as claimed in any of the preceding
20 claims wherein the replacement nucleic acid
21 sequence comprises amino acid codons which encode
22 at least part of the gene product, and differ from
23 the gene to be suppressed in at least the third
24 base of at least one of the codons.
25
- 26 5. A medicament comprising either one or both of a
27 gene suppressing agent and a nucleic acid encoding
28 at least part of a replacement gene product for
29 use in a strategy as claimed in any of the
30 preceding claims.
31
- 32 6. A medicament comprising a nucleic acid sequence
33 encoding at least part of a gene product wherein
34 the sequence differs from an endogenous gene in at
35 least the third base of at least one amino acid
36 codon.

- 1 7. A strategy for suppressing specifically or
2 partially specifically an endogenous gene and
3 introducing a replacement gene, said strategy
4 comprising the steps of:
5
6 1. providing suppressing nucleic acids or other
7 suppression effectors able to bind to an
8 endogenous gene, gene transcript or gene
9 product to be suppressed and
10
11 2. providing genomic DNA or cDNA (complete or
12 partial) encoding a replacement gene wherein
13 the suppressing nucleic acids are unable to
14 bind to equivalent regions in the genomic DNA
15 or cDNA to prevent expression of the
16 replacement gene.
17
18 8. A strategy as claimed in Claim 8 wherein the
19 coding sequence of replacement nucleic acids has
20 been altered to prevent or reduce efficiency of
21 suppression.
22
23 9. A strategy as claimed in Claim 8 or 9 wherein
24 replacement nucleic acids have modifications in
25 one or more third base (wobble) positions such
26 that replacement nucleic acids still code for the
27 wild type or equivalent amino acids.

Diagram 1

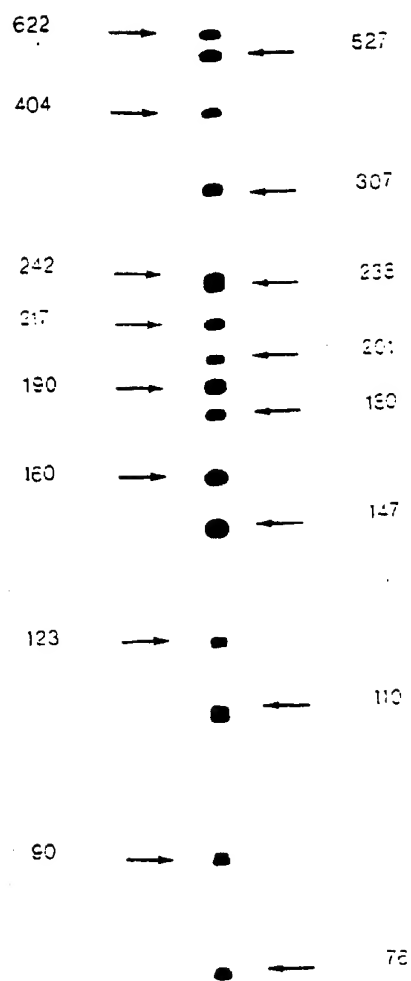


Figure 1 A + B

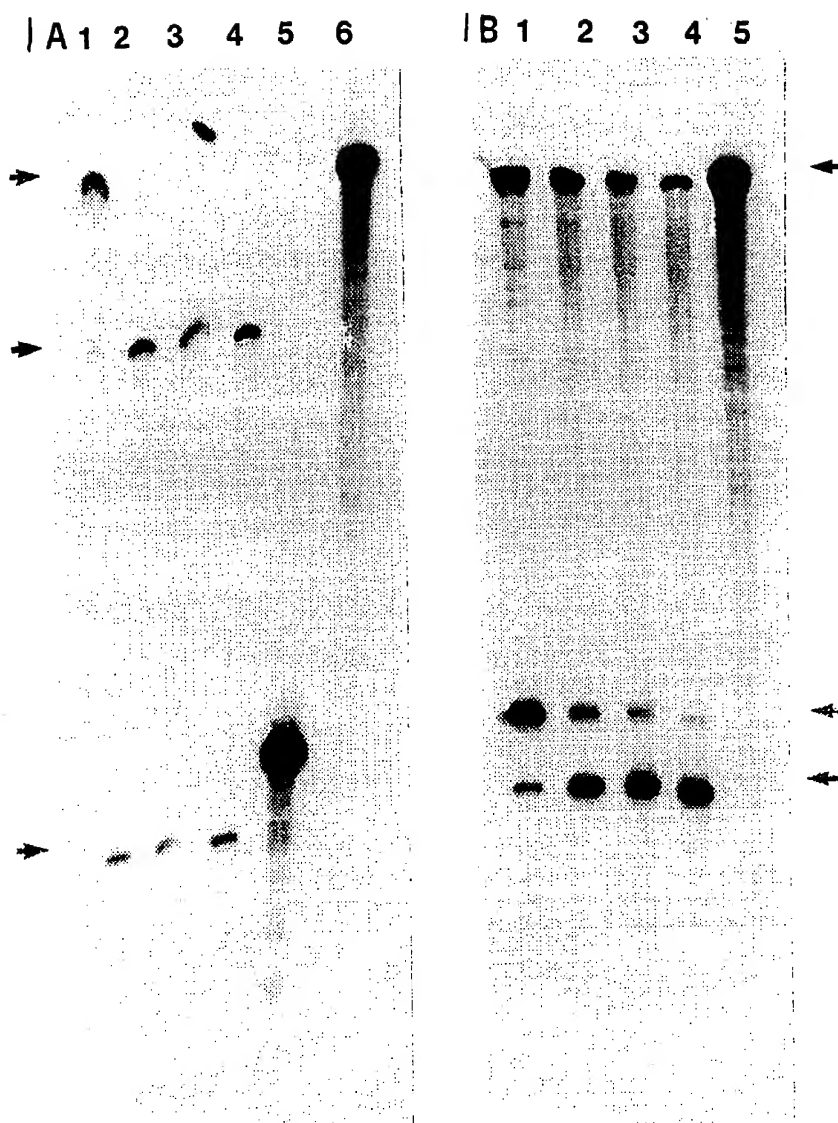
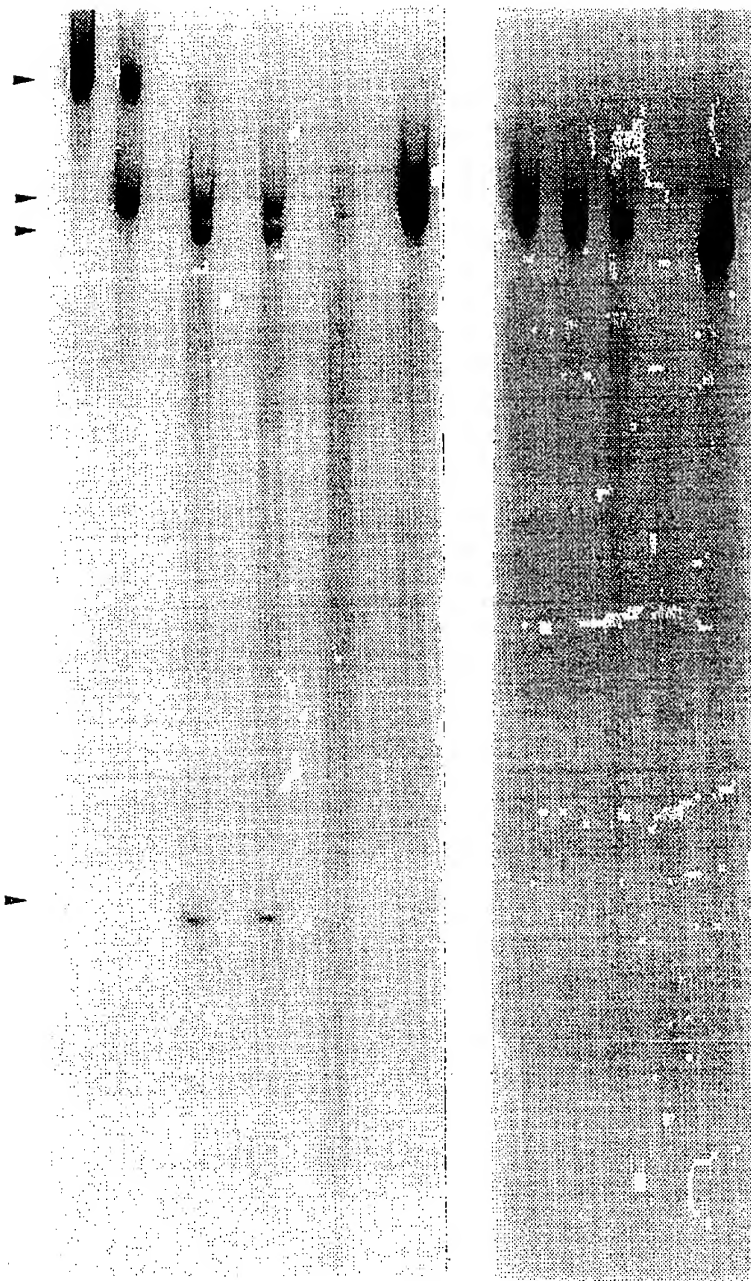


Figure 2 A+B

2 A 1 2 3 4 5 6 2B 1 2 3 4 5



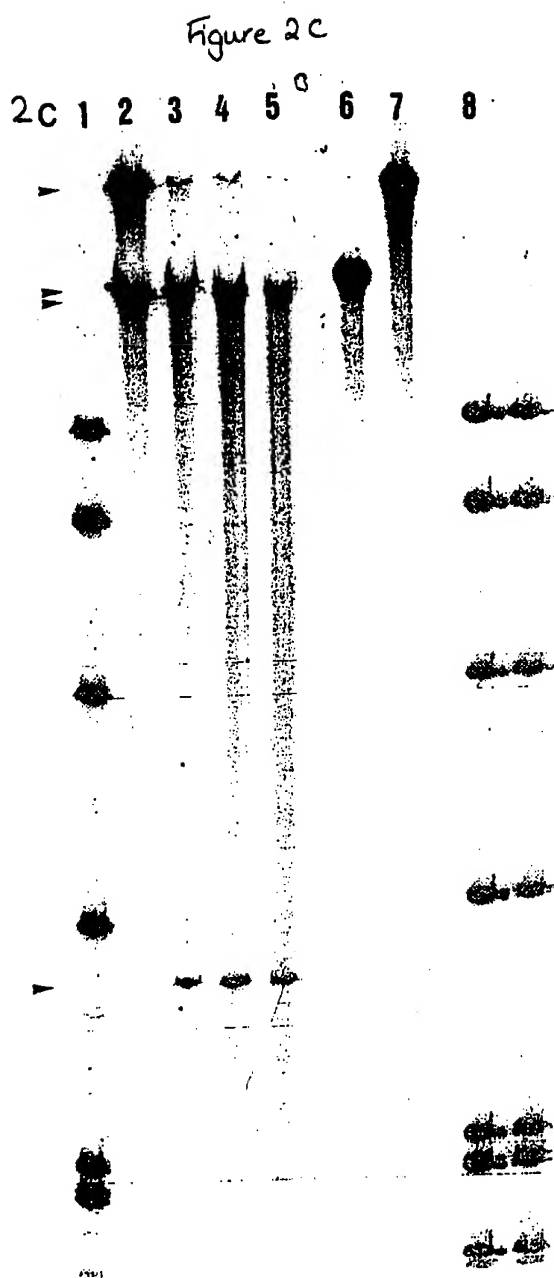


Figure 3

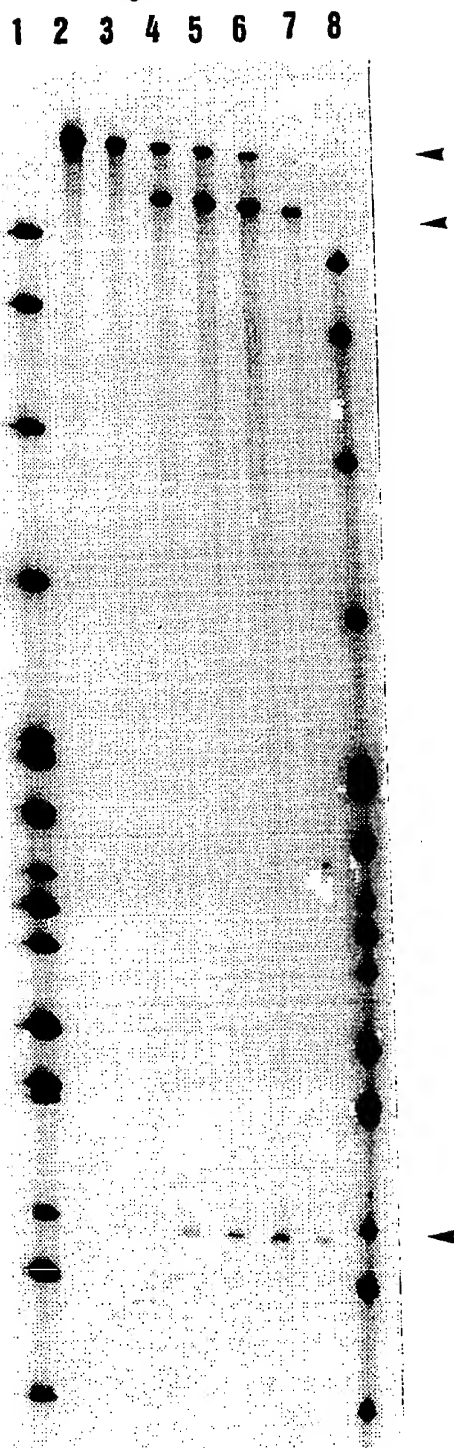


Figure 4

1 2 3 4 5 6 7 8

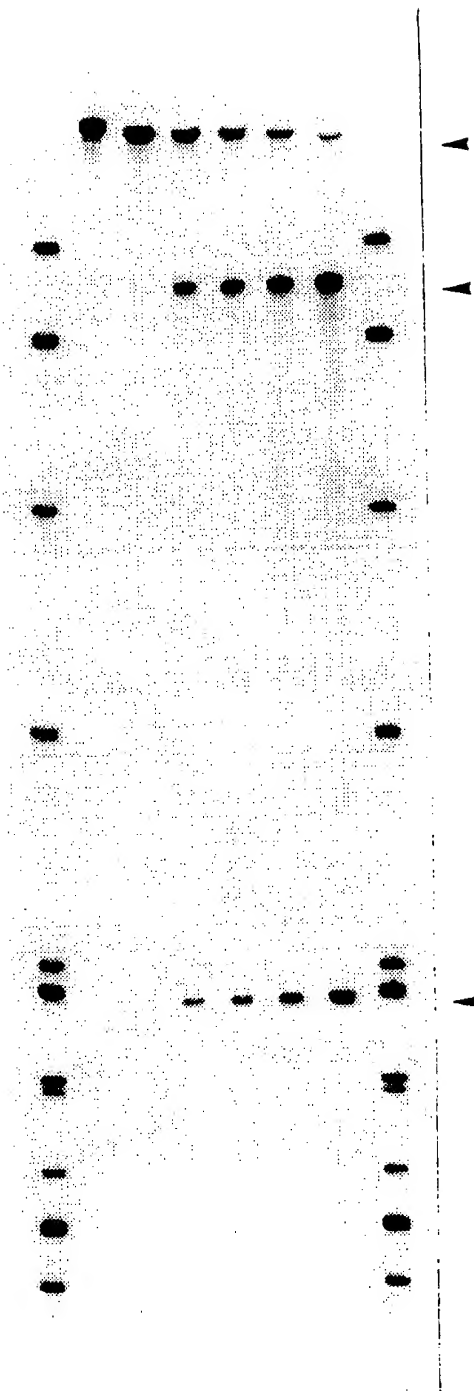


Figure 5 A

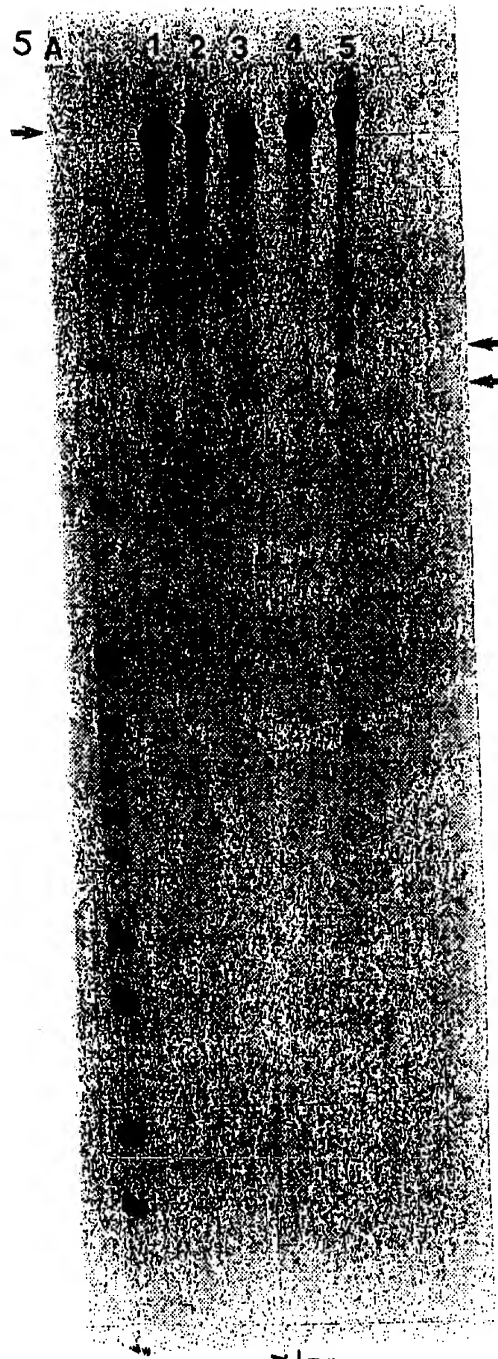


Figure 5 B

5 8 1 2 3 4 5 6

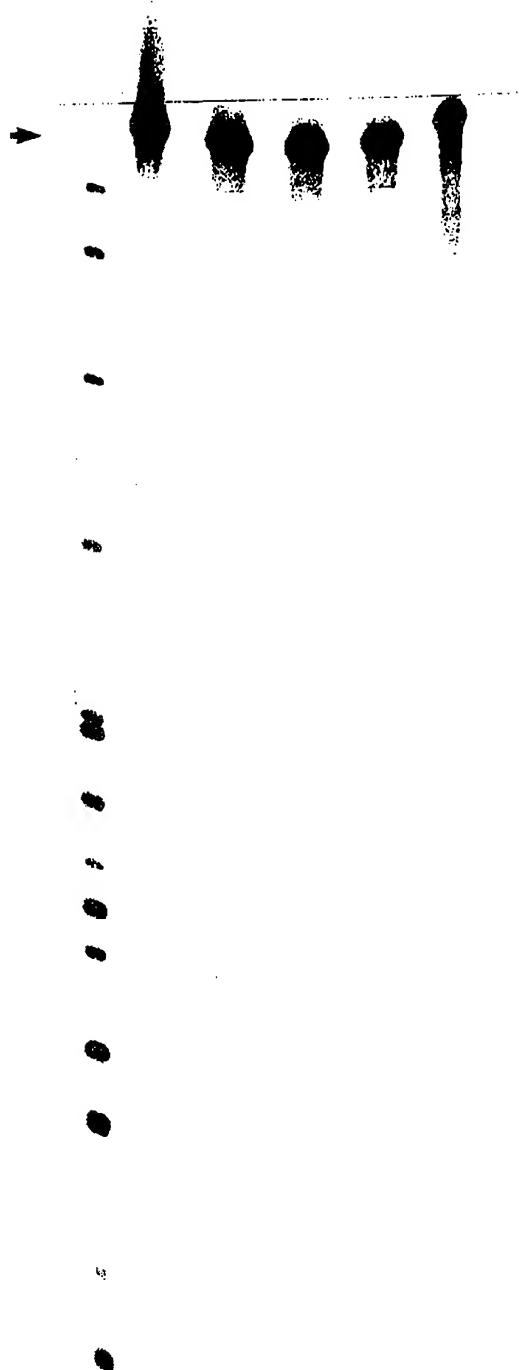


Figure 6 A+B

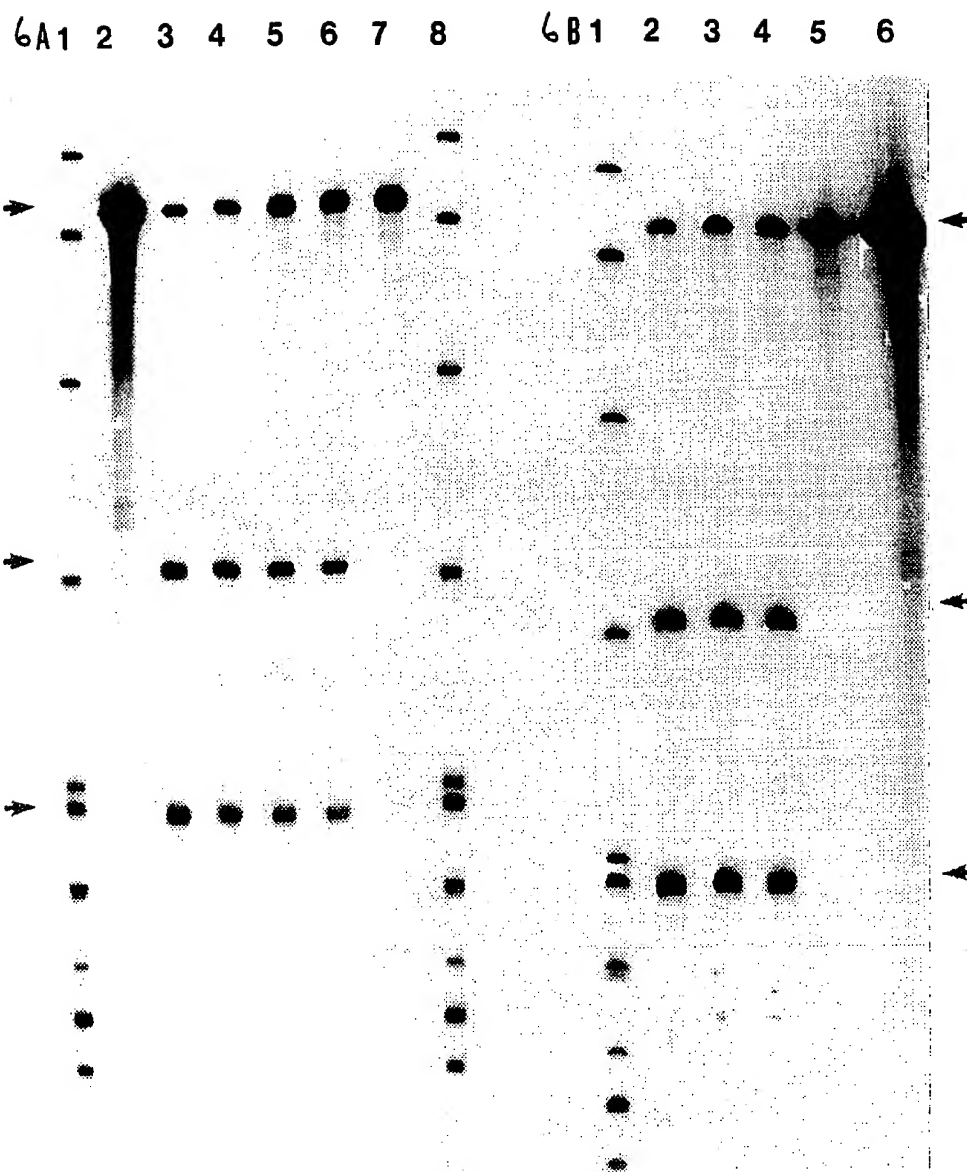


Figure 6C



Figure 6D

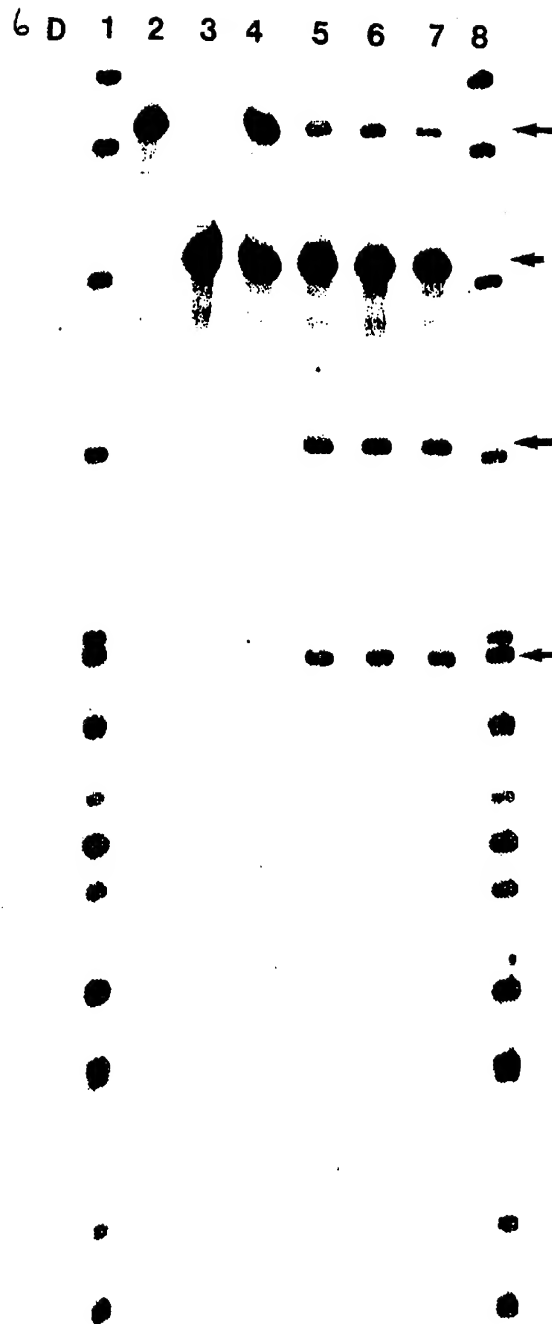
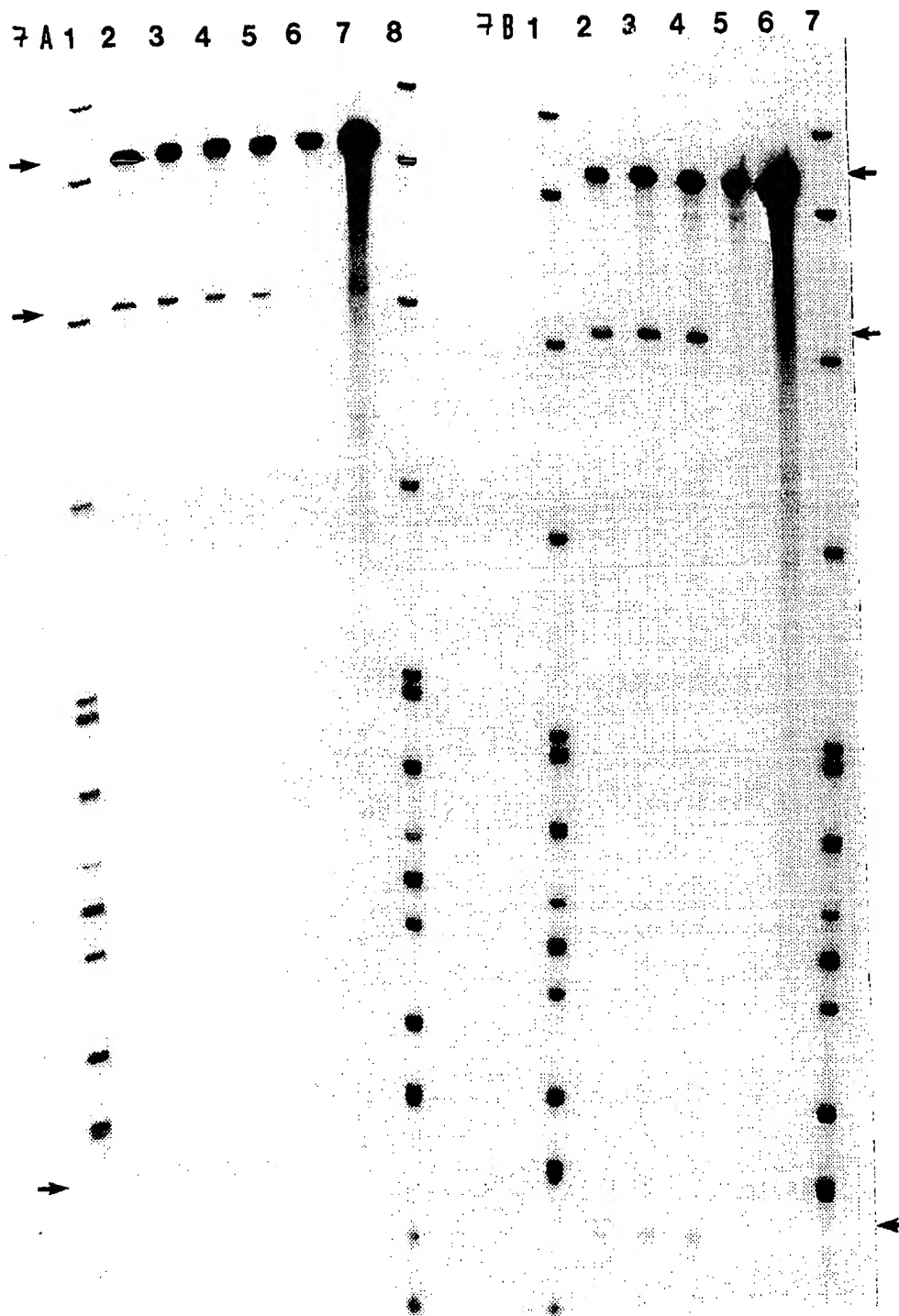


Figure 7 A + B



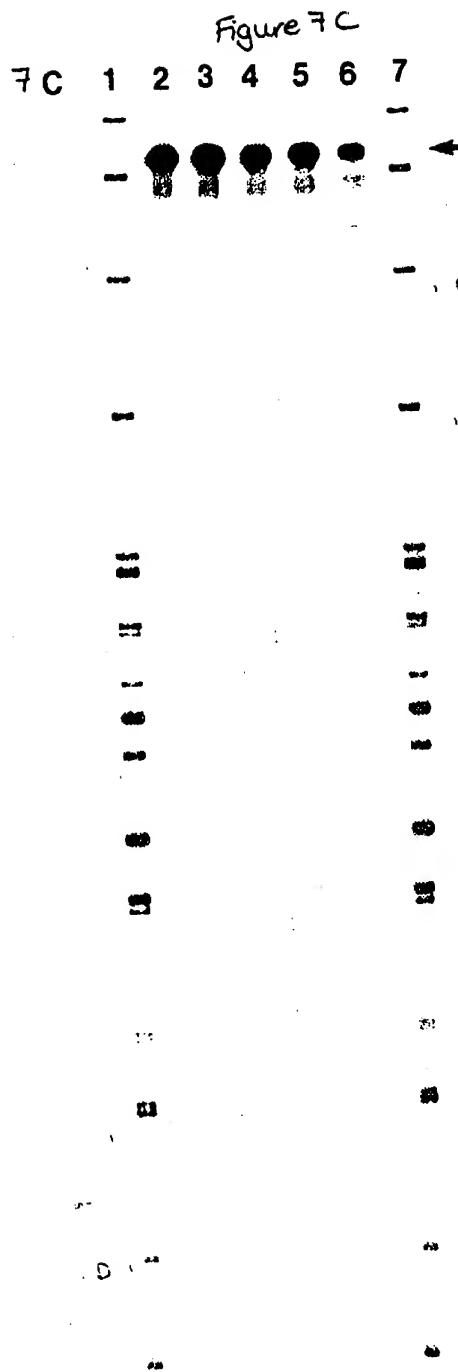
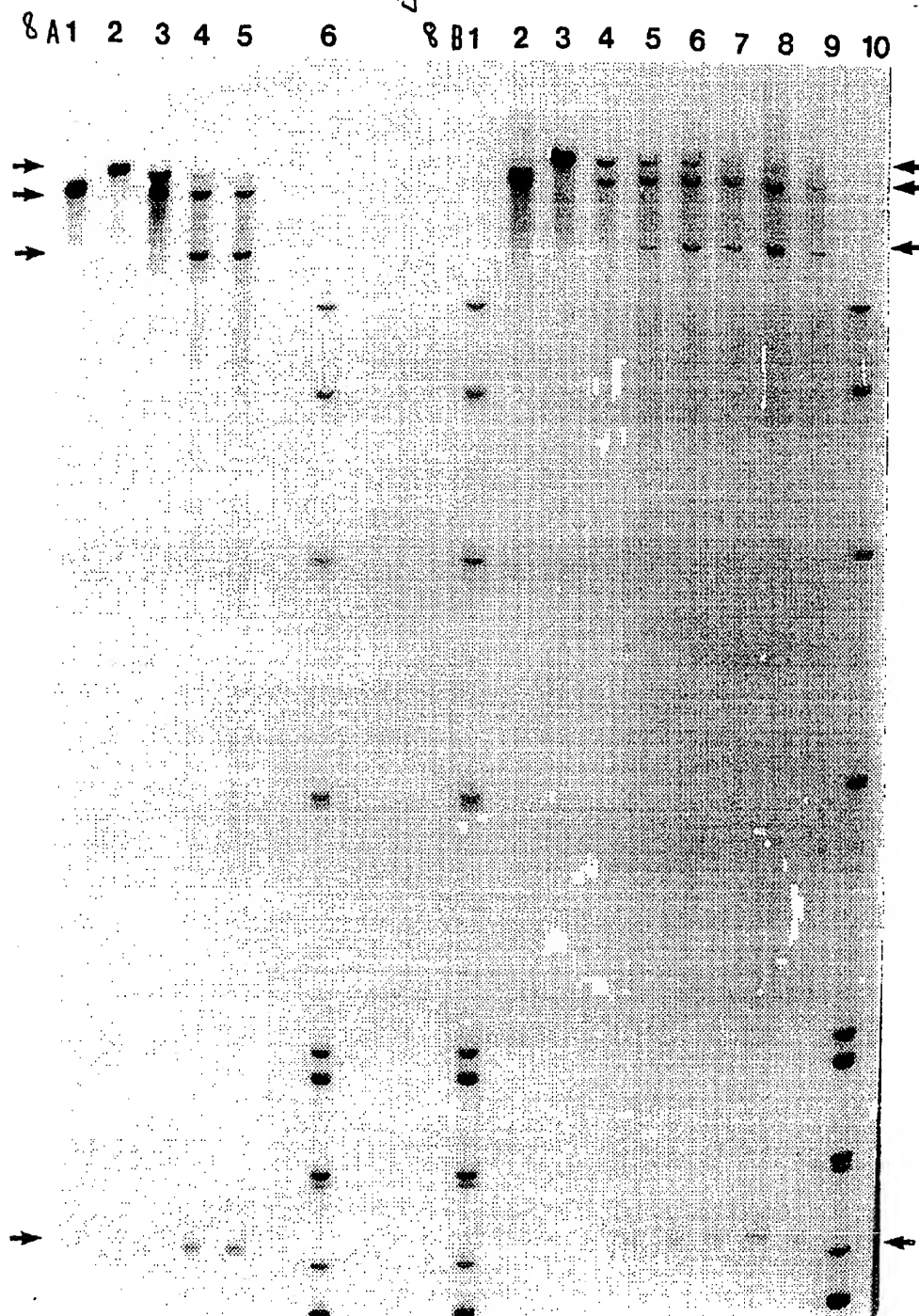
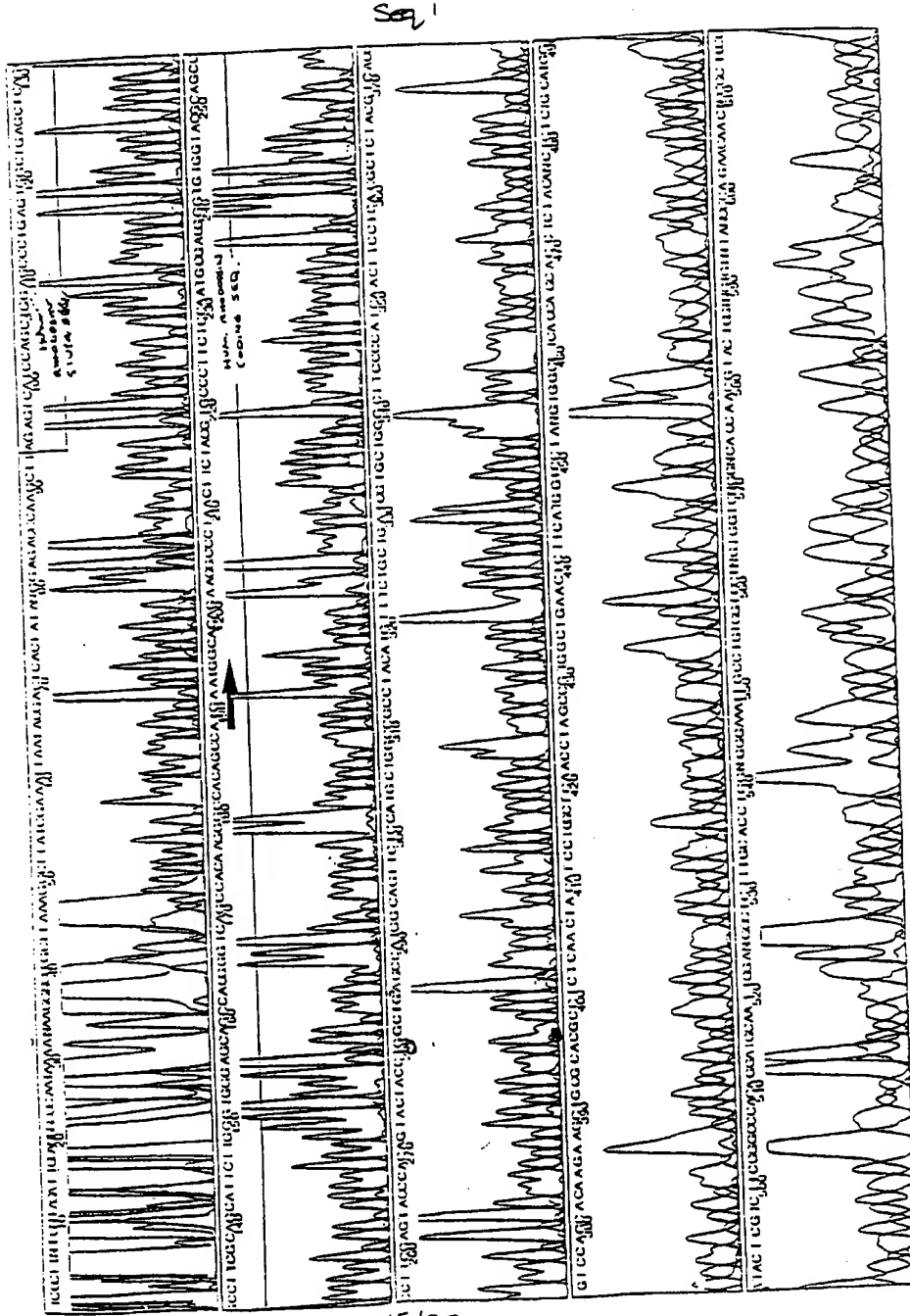


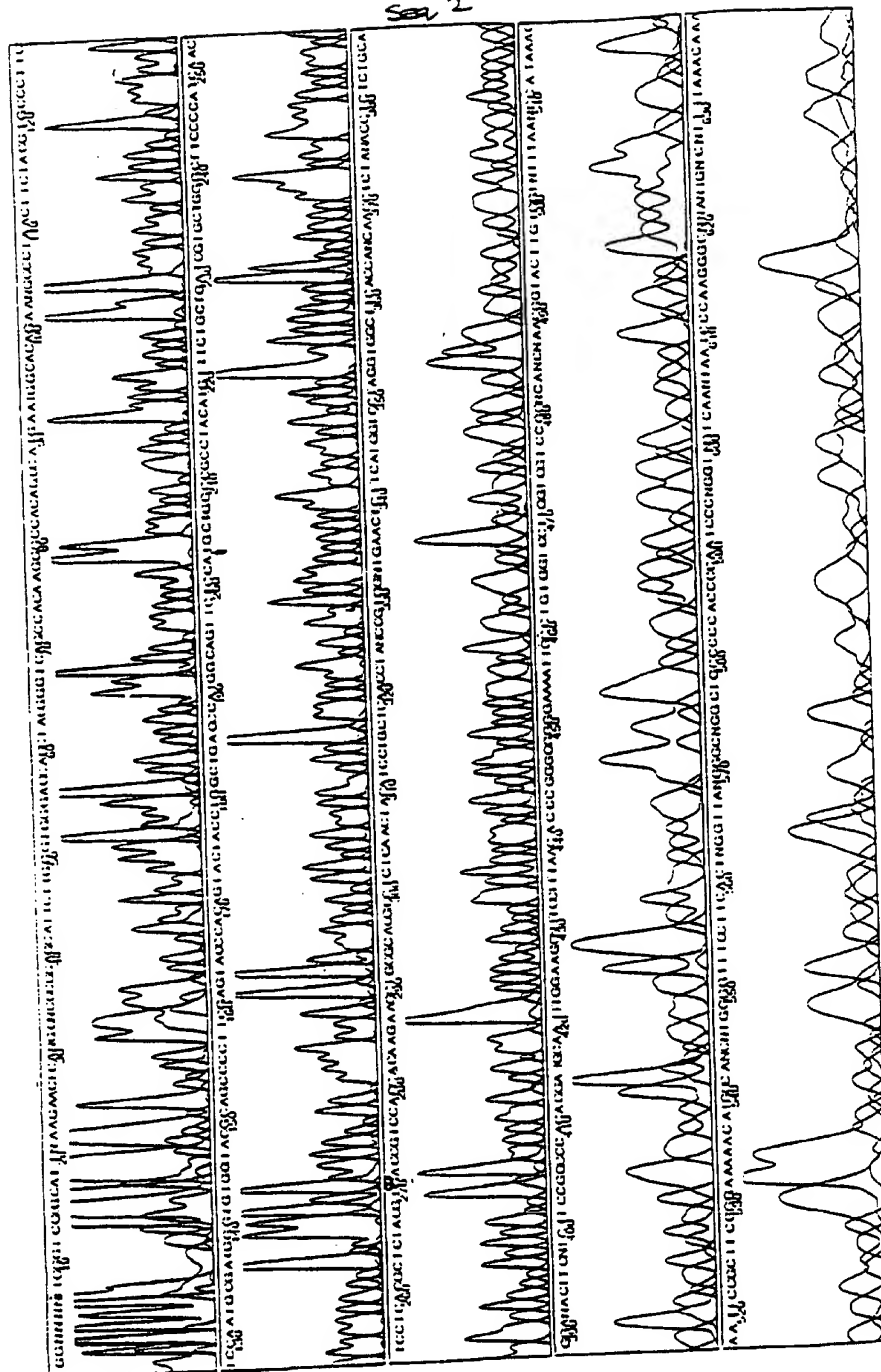
Figure 8 A+B





S
1

5
30



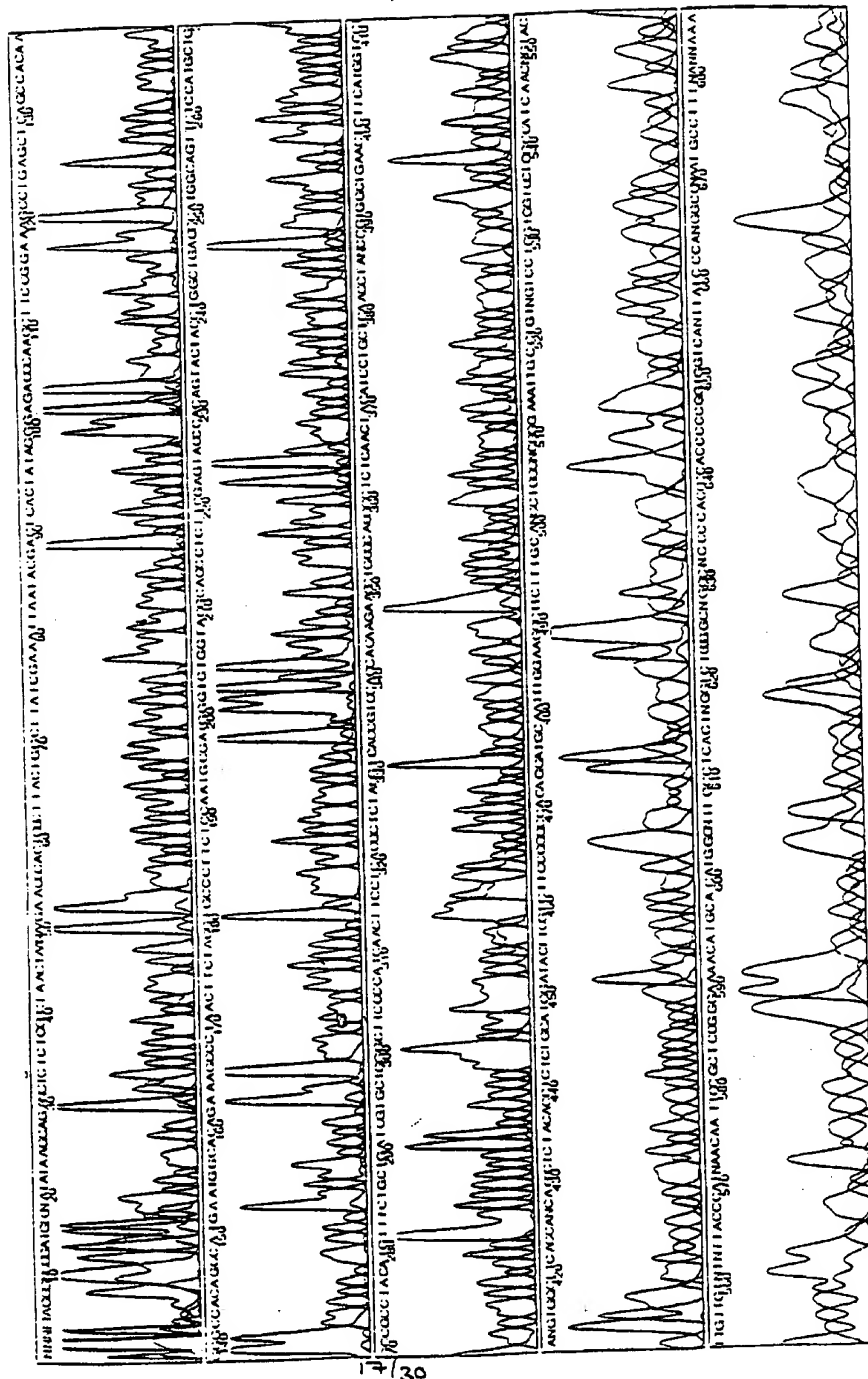
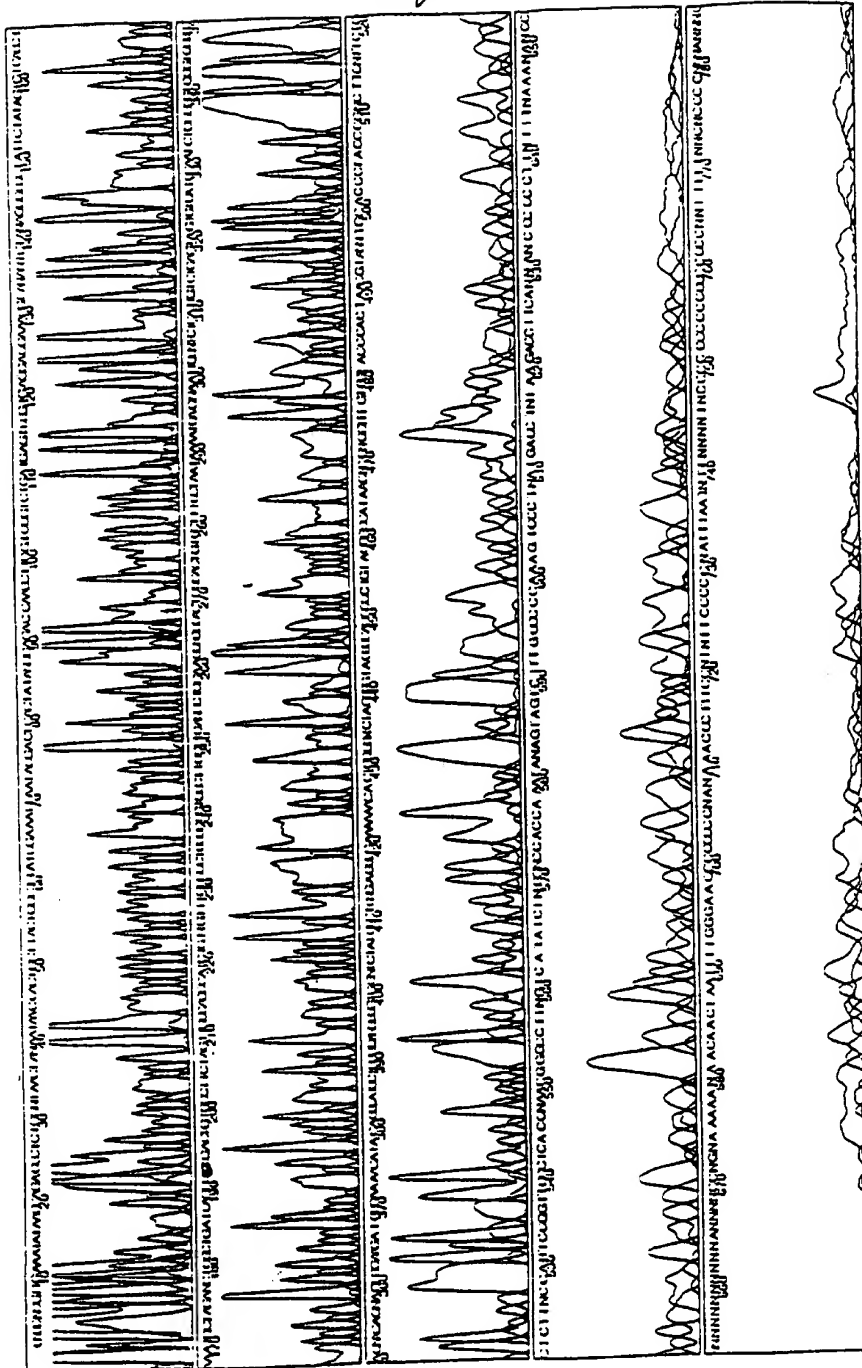
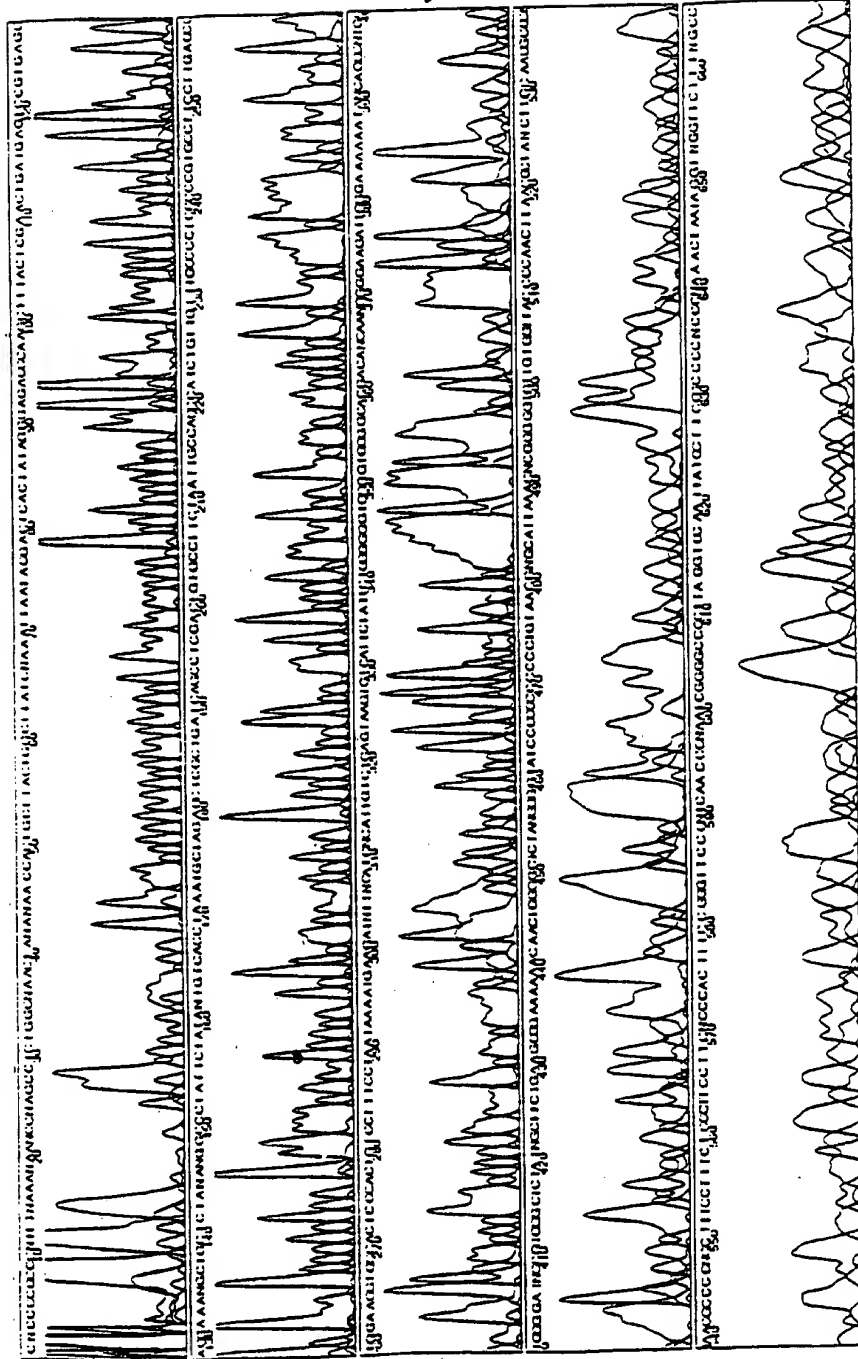


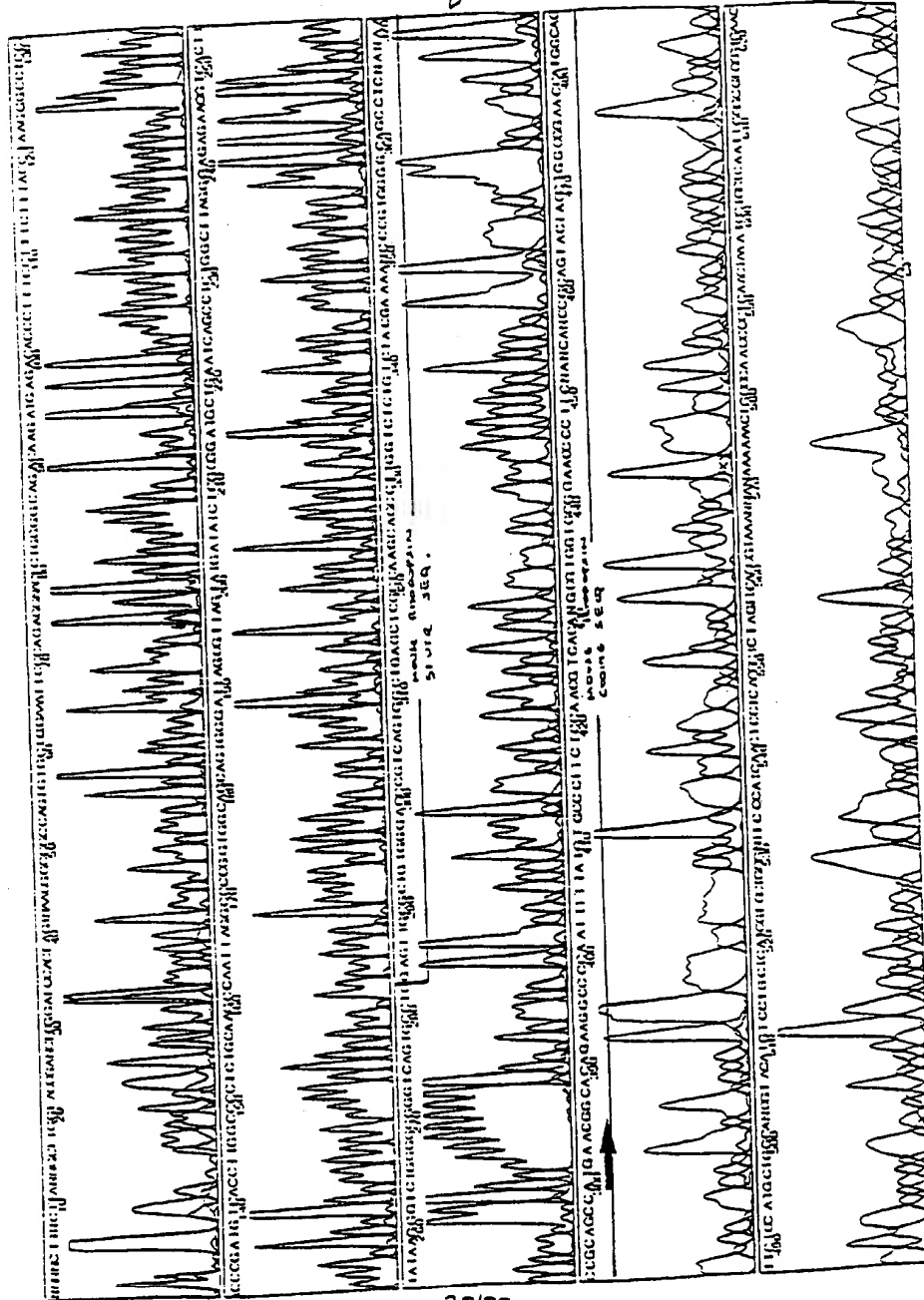
Fig. 4



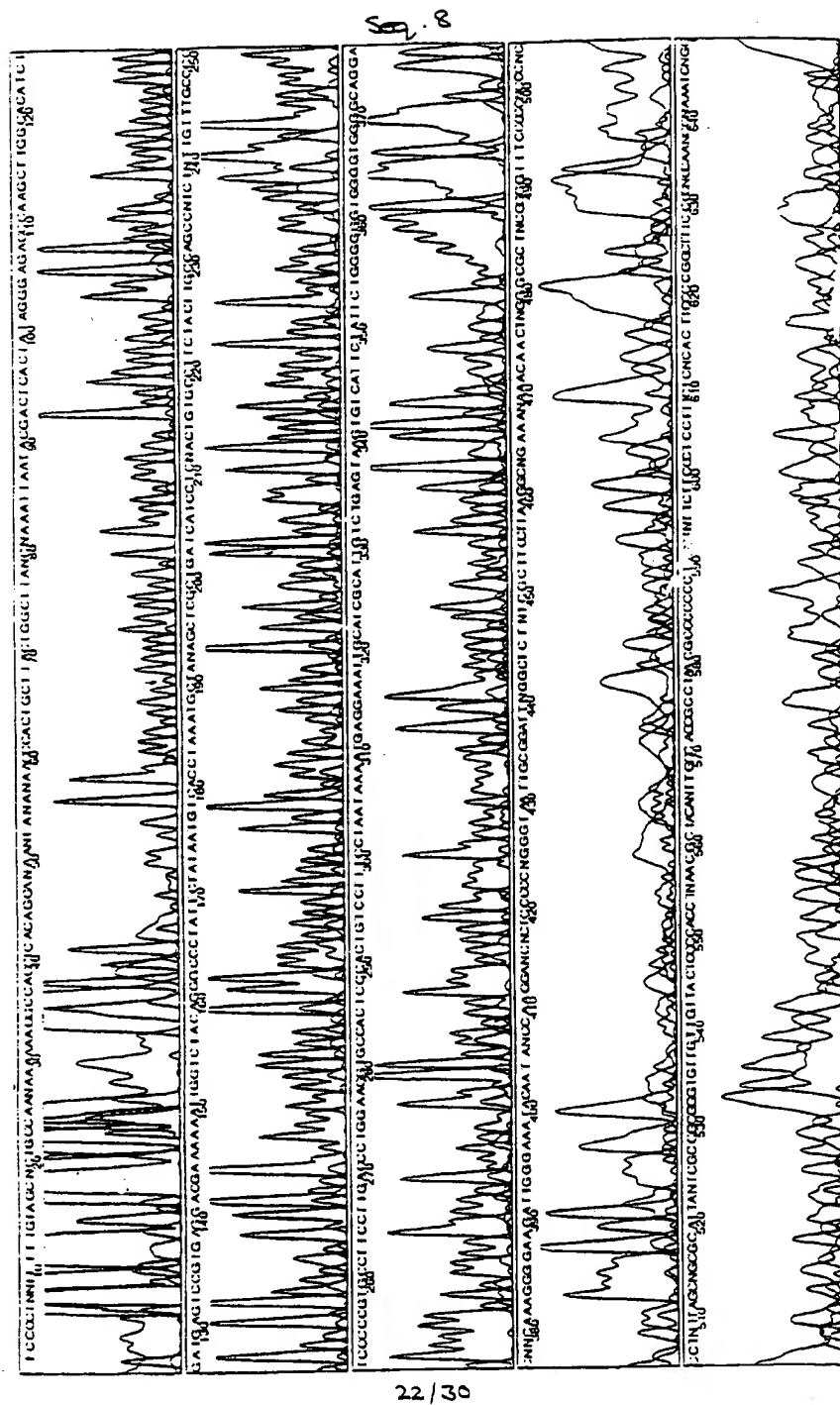


5

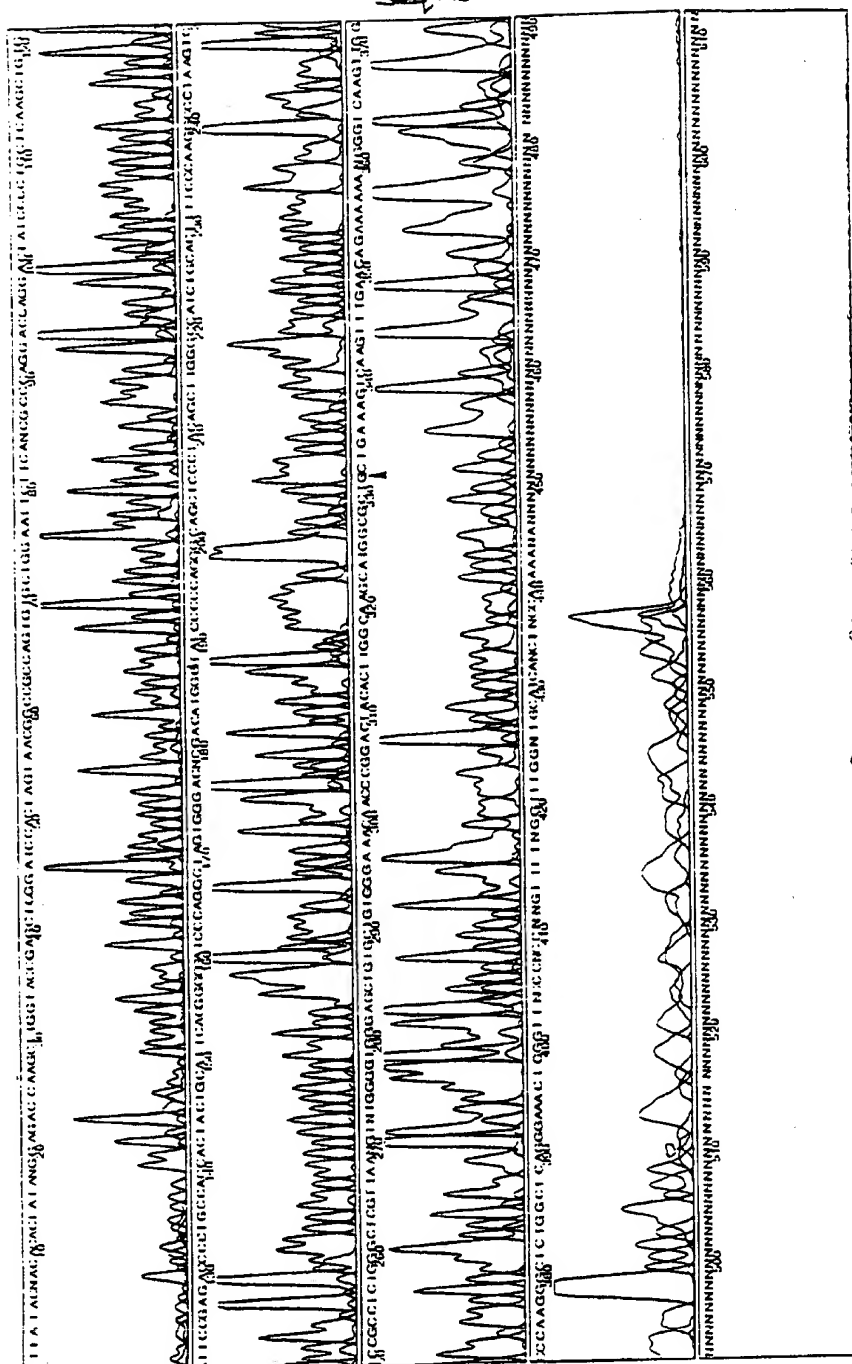
Seq. 6











10

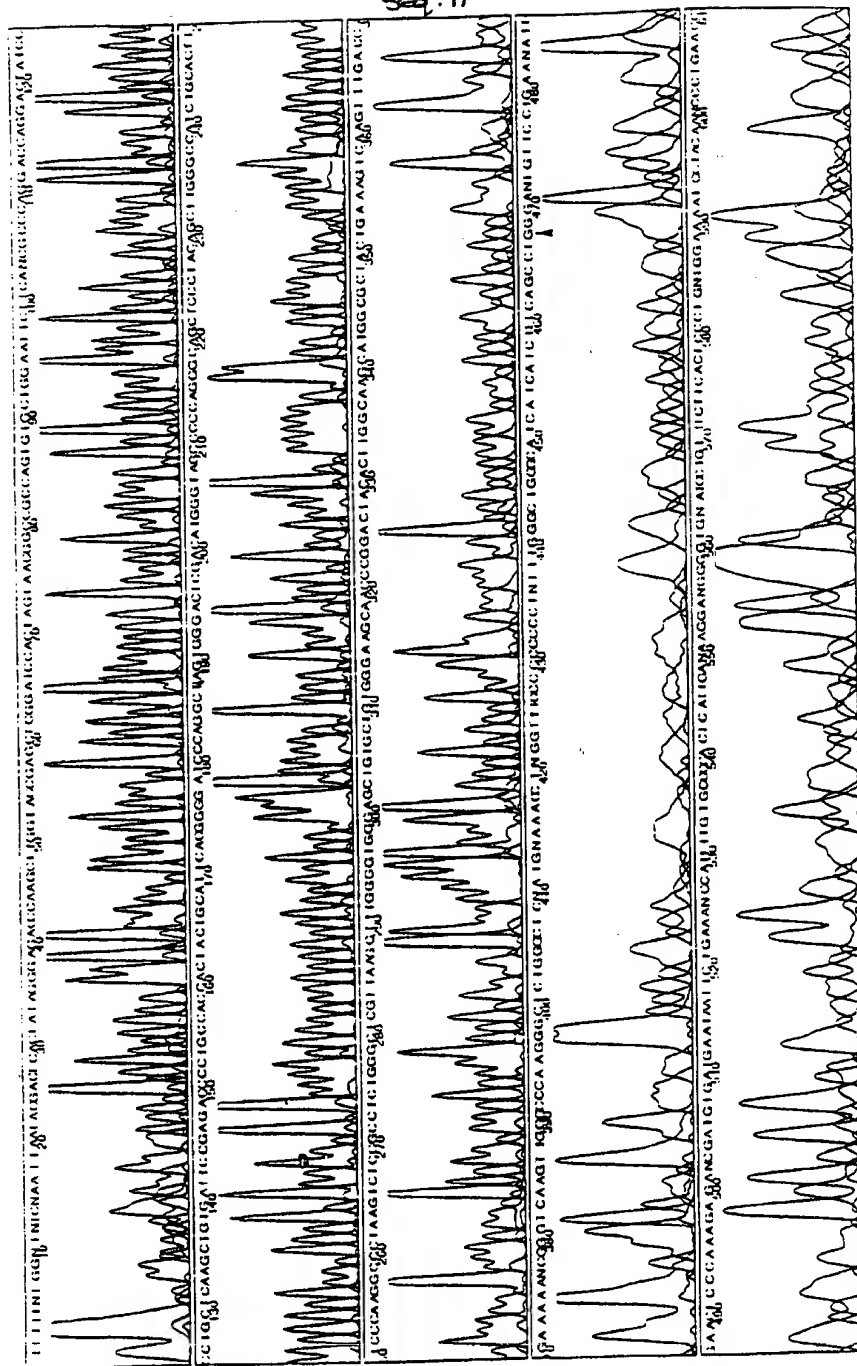
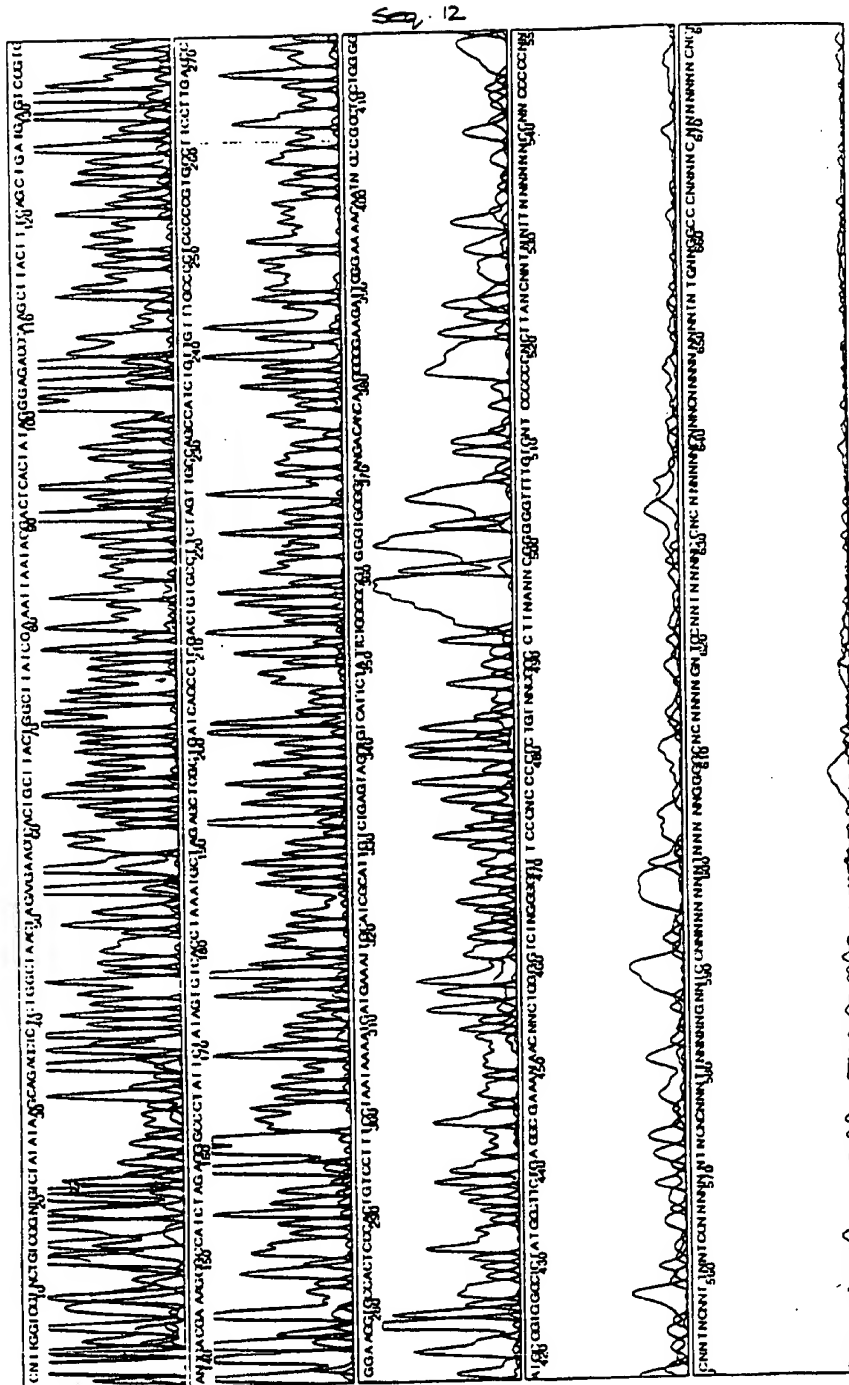
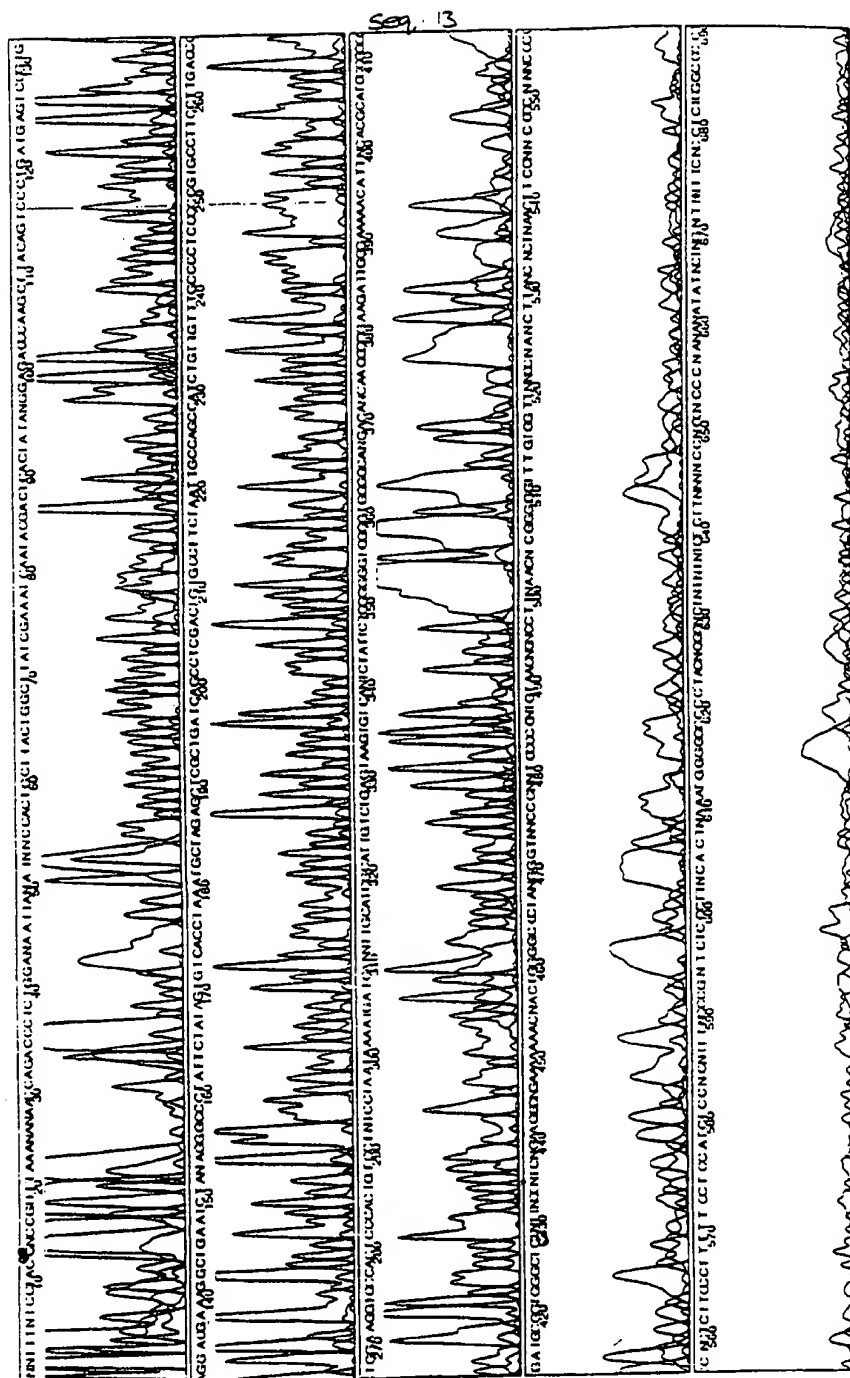
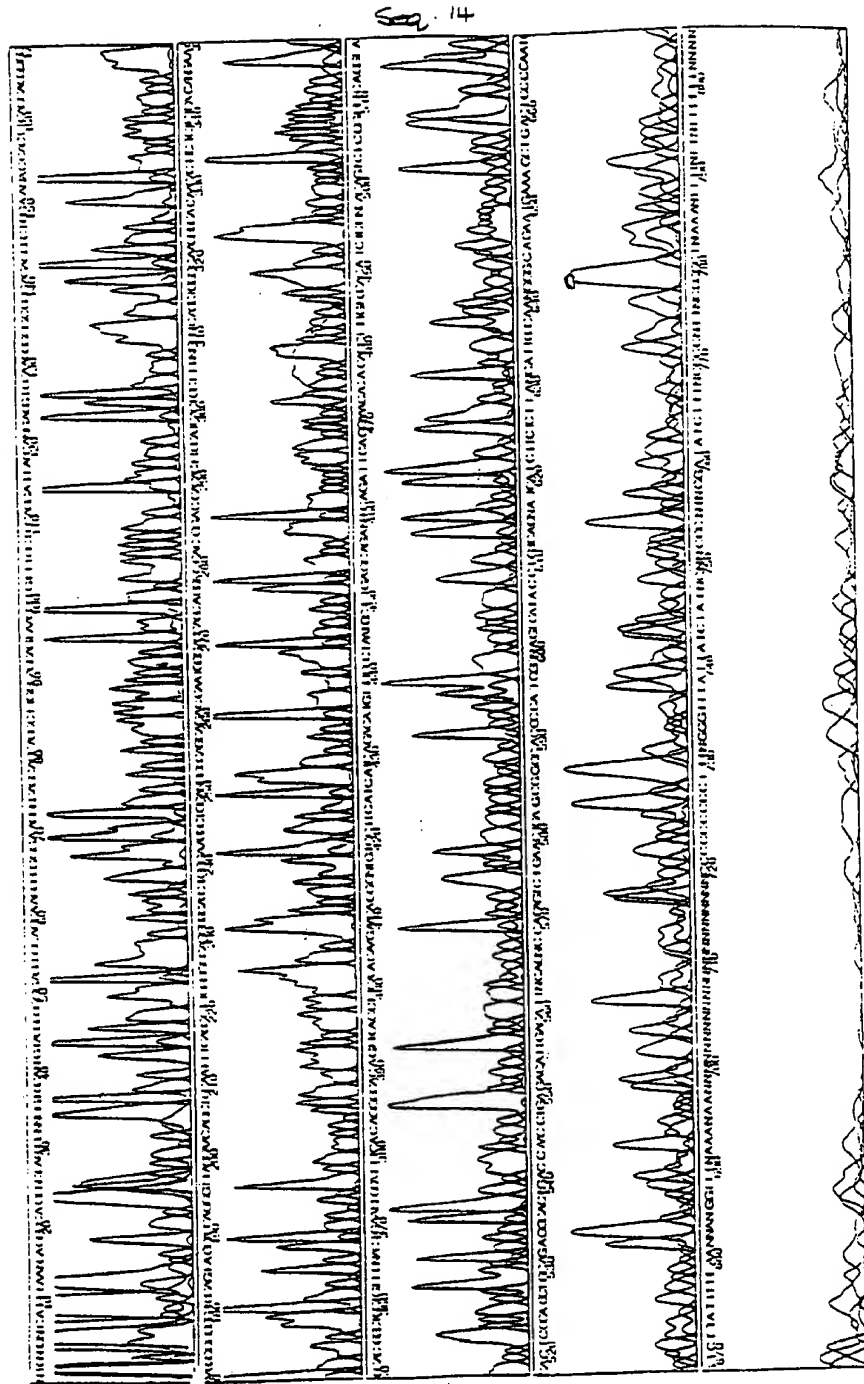
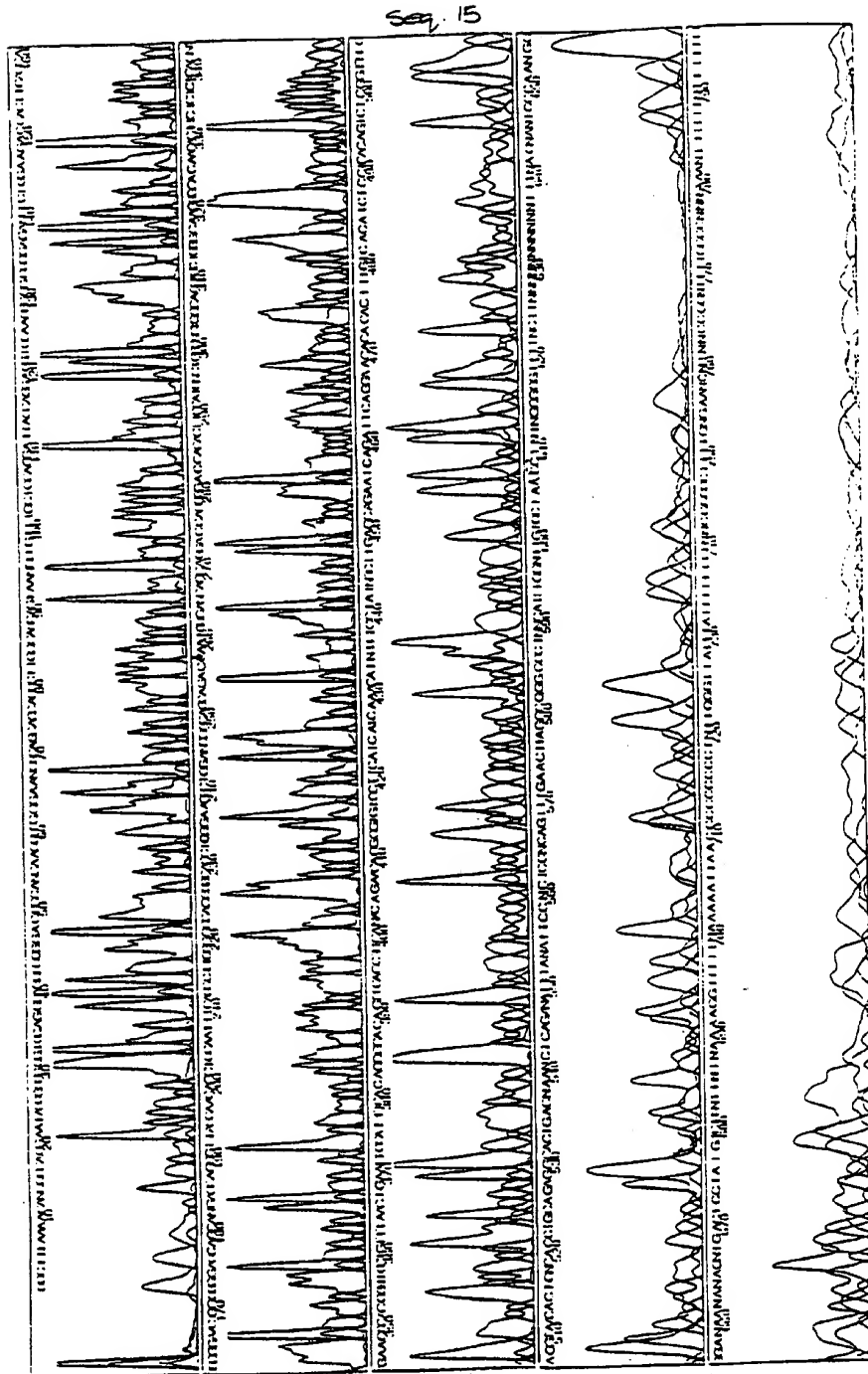


Fig. 11

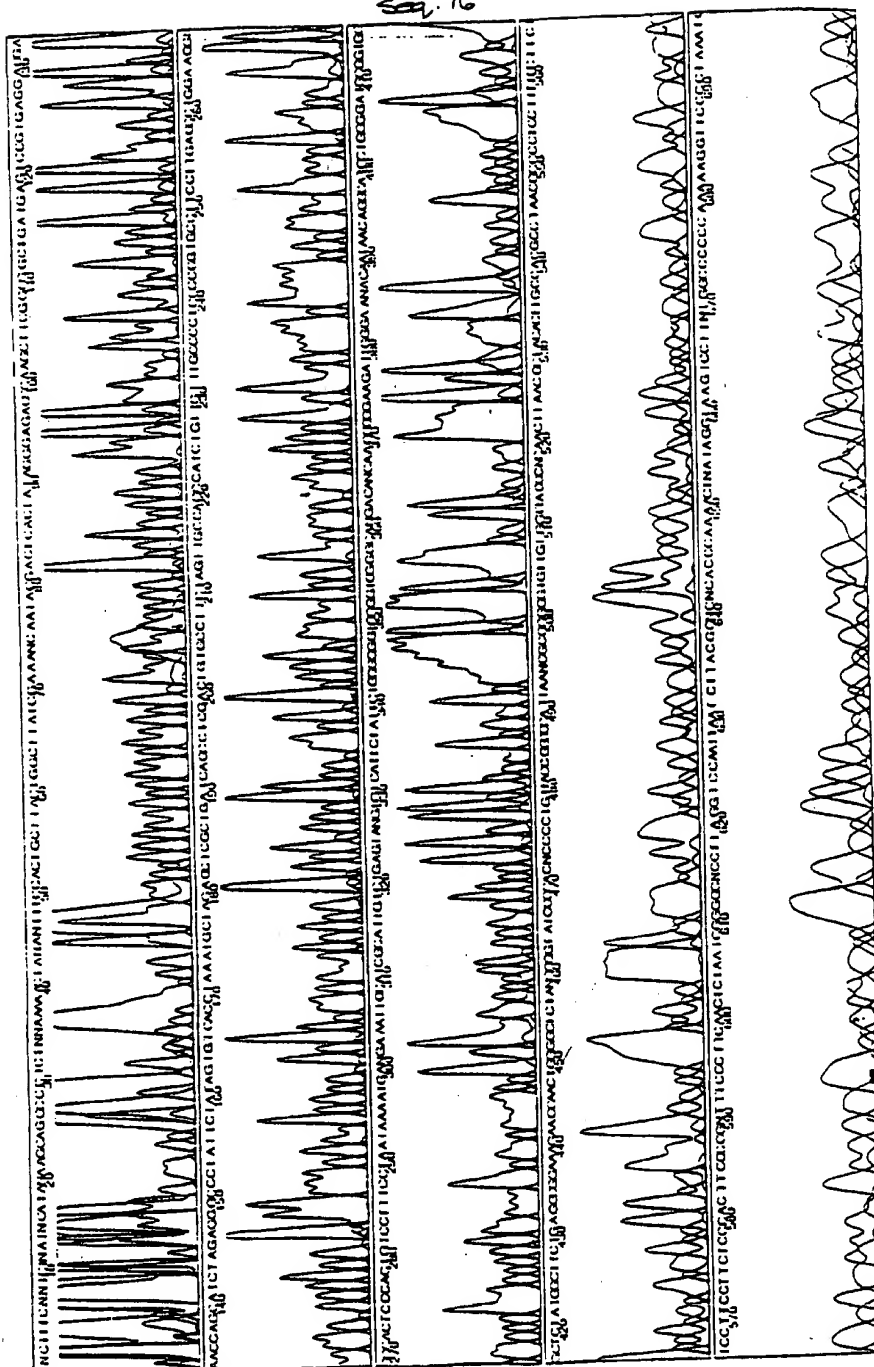








8.15



INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 97/00929

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/11 C12N9/00 A61K48/00 A61K31/70 C07H21/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N A61K C07H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LEUKEMIA, vol. 8, no. SUPPL. 01, April 1994, pages 152-155, XP000652853 ROBINSON-BENION C ET AL: "GENE TRANSPLANTATION: COMBINED ANTISENSE INHIBITION AND GENE REPLACEMENT STRATEGIES"	1-3,7,8
Y	see the whole document	4,9
Y	HUMAN MOLECULAR GENETICS, (1995 SEP) 4 (9) 1597-602., XP002038178 HART S L ET AL: "The introduction of two silent mutations into a CFTR cDNA construct allows improved detection of exogenous mRNA in gene transfer experiments." see the whole document	4,9
	--- -/-	

☒ Further documents are listed in the continuation of box C.

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Date of the actual completion of the international search

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Date of mailing of the international search report

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Andres, S

INTERNATIONAL SEARCH REPORT

International Application No
PC1/GB 97/00929

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 475 623 A (UNIV CALIFORNIA) 18 March 1992 see page 7, line 37 - line 45	5,6
A	see page 8, line 6 - line 25 ---	4,7-9
X	US 5 240 846 A (COLLINS FRANCIS S ET AL) 31 August 1993 see column 3, line 3 - line 8 see column 6, line 58 - column 7, line 8 see example I ---	5,6
A	WO 93 12257 A (HYBRITECH INC) 24 June 1993 see page 8, line 12 - line 33 see page 32, line 30 - page 33, line 24 see page 36; example III ---	4,9
A	WO 94 11494 A (UNIV JEFFERSON ;PROCKOP DARWIN (US); COLIGE ALAIN (BE); BASERGA RE) 26 May 1994 ---	
P,X	WO 97 11169 A (TRINITY COLLEGE DUBLIN ;FARRAR GWENYTH JANE (IE); HUMPHRIES PETER) 27 March 1997 see page 6, line 25 - page 11, line 5 see examples -----	1-3,7,8

INTERNATIONAL SEARCH REPORT

International application No.

PC 1/GB 97/00929

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

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because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claim(s) 1-4, 7-9
is(are) directed to a method of treatment of the human/animal
body, the search has been carried out and based on the alleged
effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such
an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all
searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment
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3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report
covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is
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Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PC/GB 97/00929

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0475623 A	18-03-92	AT 136793 T	15-05-96
		AU 8183694 A	23-02-95
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		US 5512463 A	30-04-96
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WO 9711169 A	27-03-97	AU 7089696 A	09-04-97

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(51) International Patent Classification ⁶: C12N 15/11, 9/00, A61K 48/00, 31/70, C07H 21/00	A1	(11) International Publication Number: WO 97/37014 (43) International Publication Date: 9 October 1997 (09.10.97)
(21) International Application Number: PCT/GB97/00929 (22) International Filing Date: 2 April 1997 (02.04.97) (30) Priority Data: 9606961.2 2 April 1996 (02.04.96) GB (71) Applicant (for all designated States except US): PROVOST, FELLOWS AND SCHOLARS OF THE COLLEGE OF THE HOLY AND UNDIVIDED TRINITY OF QUEEN ELIZABETH NEAR DUBLIN [IE/IE]; Trinity College Dublin, Dublin 2 (IE). (72) Inventors; and (75) Inventors/Applicants (for US only): FARRAR, Gwenth, Jane [IE/IE]; 9 The Crescent, Monkstown D20, County Dublin (IE). HUMPHRIES, Peter [GB/IE]; 5 Holmwood, Cabinteely D15, County Dublin (IE). KENNA, Paul, Francis [IE/IE]; 176 New Cabra Road, Dublin 7 (IE). (74) Agent: MURGITROYD & COMPANY; 373 Scotland Street, Glasgow G5 8QA (GB).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: GENETIC SUPPRESSION AND REPLACEMENT (57) Abstract A strategy for suppressing specifically or partially specifically an endogenous gene and introducing a replacement gene, said strategy comprising the steps of: 1) providing suppressing nucleic acids or other suppression effectors able to bind to an endogenous gene, gene transcript or gene product to be suppressed, and 2) providing genomic DNA or cDNA (complete or partial) encoding a replacement gene wherein the suppressing nucleic acids are unable to bind to equivalent regions in the genomic DNA or cDNA to prevent expression of the replacement gene. The replacement nucleic acids have modifications in one or more third base (wobble) positions such that replacement nucleic acids still code for the wild type or equivalent amino acids.		

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1 **"Genetic Suppression and Replacement"**

2

3 The present invention relates to a strategy for
4 suppressing a gene. In particular the invention
5 relates to suppression of mutated genes which give rise
6 to a dominant or deleterious effect, either
7 monogenically or polygenically. The invention relates
8 to a strategy for suppressing a gene or disease allele
9 using methods which do not target the disease allele
10 specifically but instead can be targeted towards a
11 broad range of sequences in a particular gene. A
12 particular embodiment of the invention is the use of
13 suppression strategies to target either the disease or
14 normal alleles alone or to target both disease and
15 normal alleles. A further embodiment of the invention
16 is the use of the wobble hypothesis to enable continued
17 expression of a replacement normal or beneficial gene
18 (a gene modified from the wild type such that it
19 provides an additional beneficial effect(s)). The
20 replacement gene will have nucleotide changes from the
21 endogenous wild type gene but will code for identical
22 amino acids as the wild type gene. The strategy
23 circumvents the need for a specific therapy for every
24 mutation within a given gene. In addition the
25 invention allows greater flexibility in choice of

1 target sequence for suppression of a disease allele.

2

3 The invention also relates to a medicament or
4 medicaments for use in suppressing a deleterious allele
5 which is present in a genome of one or more individuals
6 or animals.

7

8 Generally the present invention will be useful where
9 the gene, which is naturally present in the genome of a
10 patient, contributes to a disease state. Generally,
11 one allele of the gene in question will be mutated,
12 that is, will possess alterations in its nucleotide
13 sequence that affects the function or level of the gene
14 product. For example, the alteration may result in an
15 altered protein product from the wild type gene or
16 altered control of transcription and processing.
17 Inheritance or somatic acquisition of such a mutation
18 can give rise to a disease phenotype or can predispose
19 an individual to a disease phenotype. However the gene
20 of interest could also be of wild type phenotype, but
21 contribute to a disease state in another way such that
22 the suppression of the gene would alleviate or improve
23 the disease state or improve the effectiveness of an
24 administered therapeutic compound.

25

26 Generally, suppression effectors such as nucleic acids
27 - antisense or sense, ribozymes, peptide nucleic acids
28 (PNAs), triple helix forming oligonucleotides, peptides
29 and /or antibodies directed to sequences in a gene, in
30 transcripts or in protein, can be employed in the
31 invention to achieve gene suppression.

32

33 BACKGROUND

34

35 Studies of degenerative hereditary ocular conditions,
36 including Retinitis Pigmentosa (RP) and various macular

1 dystrophies have resulted in a substantial elucidation
2 of the molecular basis of these debilitating human
3 retinal degenerations. Applying the approach of
4 genetic linkage, x-linked RP (xLRP) genes have been
5 localised to the short arm of the X chromosome (Ott et
6 al. 1990) - subsequently the gene involved in one form
7 of xLRP has been identified. Various genes involved in
8 autosomal dominant forms of RP (adRP) have been
9 localised. The first of these mapped on 3q close to
10 the gene encoding the rod photoreceptor protein
11 rhodopsin (McWilliam et al. 1989; Dryja et al. 1990).
12 Similarly, an adRP gene was placed on 6p close to the
13 gene encoding the photoreceptor protein peripherin
14 (Farrar et al. 1991a,b; Kajiwara et al. 1991).
15 Other adRP genes have been mapped to discrete
16 chromosomal locations however the disease genes as yet
17 remain uncharacterised. As in xLRP and adRP, various
18 genes involved in autosomal recessive RP (arRP) have
19 been localised and in some cases molecular defects
20 characterised (Humphries et al. 1992; Farrar et al.
21 1993; Van Soest et al. 1994). Similarly a number of
22 genes involved in macular dystrophies have been mapped
23 (Mansergh et al. 1995). Genetic linkage, together with
24 techniques for mutational screening of candidate genes,
25 enabled identification of causative dominant mutations
26 in the genes encoding rhodopsin and peripherin.
27 Globally about 100 rhodopsin mutations have been found
28 in patients with RP or congenital stationary night
29 blindness. Similarly approximately 40 mutations have
30 been characterised in the peripherin gene in patients
31 with RP or macular dystrophies. Knowledge of the
32 molecular aetiology of these retinopathies has
33 stimulated the generation of animal models and the
34 exploration of methods of therapeutic intervention
35 (Farrar et al. 1995; Humphries et al. 1997).
36

1 Similar to RP, osteogenesis imperfecta (OI) is an
2 autosomal dominantly inherited human disorder whose
3 molecular pathogenesis is extremely genetically
4 heterogeneous. OI is often referred to as 'brittle
5 bone disease' although additional symptoms including
6 hearing loss, growth deficiency, bruising, loose
7 joints, blue sclerae and dentinogenesis imperfecta are
8 frequently observed (McKusick, 1972). Mutations in the
9 genes encoding the two type I collagen chains (collagen
10 1A1 and 1A2) comprising the type I collagen heterodimer
11 have been implicated in OI. Indeed hundreds of
12 dominantly acting mutations have been identified in OI
13 patients in these two genes, many of which are single
14 point mutations, although a number of insertion and
15 deletion mutations have been found (Willing et al.
16 1993; Zhuang et al. 1996). Similarly mutations in
17 these genes have also been implicated in Ehlers-Danlos
18 and Marfan syndromes (Dalglish et al. 1986; Phillips
19 et al. 1990; D'Alessio et al. 1991; Vasan NS et al.
20 1991).

21
22 Generally, gene therapies utilising viral and non-viral
23 delivery systems have been used to treat inherited
24 disorders, cancers and infectious diseases. However,
25 many studies have focused on recessively inherited
26 disorders, the rationale being that introduction and
27 expression of the wild type gene may be sufficient to
28 prevent/ameliorate the disease phenotype. In contrast
29 gene therapy for dominant disorders will require
30 suppression of the dominant disease allele. Notably
31 many of the characterised mutations causing inherited
32 diseases such as RP or OI are inherited in an autosomal
33 dominant fashion. Indeed there are over 1,000
34 autosomal dominantly inherited disorders in man. In
35 addition there are many polygenic disorders due to
36 co-inheritance of a number of genetic components

1 which together give rise to the disease state.
2 Effective gene therapies for dominant or polygenic
3 diseases may be targeted to the primary defect and in
4 this case may require suppression of the disease allele
5 while in many cases still maintaining the function of
6 the normal allele. This will be particularly relevant
7 where disease pathology is due to a gain of function
8 mutation rather than due to reduced levels of wild type
9 protein. Alternatively suppression therapies may be
10 targeted to secondary effects associated with the
11 disease pathology: one example is programmed cell death
12 (apoptosis) which has been observed in many inherited
13 disorders.

14
15 Strategies to differentiate between normal and disease
16 alleles and to selectively switch off the disease
17 allele using suppression effectors inter alia antisense
18 DNA/RNA, PNAs, ribozymes or triple helix^{forming} DNA, targeted
19 towards the disease mutation may be difficult in many
20 cases - frequently disease and normal alleles differ
21 by only a single nucleotide. A further difficulty
22 inhibiting development of gene therapies is the
23 heterogeneous nature of some dominant disorders - many
24 different mutations in the same gene give rise to a
25 similar disease phenotype. Development of specific
26 gene therapies for each of these may be prohibitive in
27 terms of cost. To circumvent difficulties associated
28 with specifically targeting the disease mutation and
29 with the genetic heterogeneity present in inherited
30 disorders, a novel strategy for gene suppression and
31 gene replacement exploiting the degeneracy of the
32 genetic code, thereby allowing flexibility in choice of
33 target sequence for suppression and providing a means
34 of gene suppression which is independent of the disease
35 mutation, is described in the invention.
36

1 PRIOR ART

2

3 Suppression effectors have been used previously to
4 achieve specific suppression of gene expression.

5 Antisense DNA and RNA has been used to inhibit gene
6 expression in many instances. Modifications, such as
7 phosphorothioates, have been made to oligonucleotides
8 to increase resistance to nuclease degradation, binding
9 affinity and uptake (Cazenave et al. 1989; Sun et al.

10 1989; McKay et al. 1996; Wei et al. 1996). In some
11 instances, using antisense and ribozyme suppression
12 strategies has led to reversal of a tumour phenotype by
13 reducing expression of a gene product or by cleaving a
14 mutant transcript at the site of the mutation (Carter
15 and Lemoine 1993; Lange et al. 1993; Valera et al.
16 1994; Dosaka-Akita et al. 1995; Feng et al. 1995;
17 Quattrone et al. 1995; Ohta et al. 1996). For example,

18 neoplastic reversion was obtained using a ribozyme
19 targeted to a H-ras mutation in bladder carcinoma cells
20 (Feng et al. 1995). Ribozymes have also been proposed
21 as a means of both inhibiting gene expression of a
22 mutant gene and of correcting the mutant by targeted
23 trans-splicing (Sullenger and Cech 1994; Jones et al.
24 1996). Ribozymes can be designed to elicit

25 autocatalytic cleavage of RNA targets, however, the
26 inhibitory effect of some ribozymes may be due in part
27 to an antisense effect due to the antisense sequences
28 flanking the catalytic core which specify the target
29 site (Ellis and Rodgers 1993; Jankowsky and Schwenzer
30 1996). Ribozyme activity may be augmented by the use
31 of, for example, non-specific nucleic acid binding
32 proteins or facilitator oligonucleotides (Herschlag et
33 al. 1994; Jankowsky and Schwenzer 1996). Multitarget
34 ribozymes (connected or shotgun) have been suggested as
35 a means of improving efficiency of ribozymes for gene
36 suppression (Ohkawa et al. 1993). Triple helix

1 approaches have also been investigated for sequence
2 specific gene suppression - triplex forming
3 oligonucleotides have been found in some cases to bind
4 in a sequence specific manner (Postel et al. 1991;
5 Duval-Valentin et al. 1992; Hardenbol and Van Dyke
6 1996; Porumb et al. 1996). Similarly peptide nucleic
7 acids have been shown to inhibit gene expression
8 (Hanvey et al. 1992; Knudson and Nielsen 1996; Taylor
9 et al. 1997). Minor groove binding polyamides can bind
10 in a sequence specific manner to DNA targets and hence
11 may represent useful small molecules for future
12 suppression at the DNA level (Trauger et al. 1996). In
13 addition, suppression has been obtained by interference
14 at the protein level using dominant negative mutant
15 peptides and antibodies (Herskowitz 1987; Rimsky et al.
16 1989; Wright et al. 1989). In some cases suppression
17 strategies have lead to a reduction in RNA levels
18 without a concomitant reduction in proteins, whereas in
19 others, reductions in RNA have been mirrored by
20 reductions in protein.

21
22 There is now an armament with which to obtain gene
23 suppression. This, in conjunction with a better
24 understanding of the molecular etiology of disease,
25 results in an ever increasing number of disease targets
26 for therapies based on suppression. In many cases,
27 complete suppression of gene expression has been
28 difficult to achieve. Possibly a combined approach
29 using a number of suppression effectors may aid in
30 this. For some disorders it may be necessary to block
31 expression of a disease allele completely to prevent
32 disease symptoms whereas for others low levels of
33 mutant protein may be tolerated. In parallel with an
34 increased knowledge of the molecular defects causing
35 disease has been the realisation that many disorders
36 are genetically heterogeneous. Examples in which

1 multiple genes and/or multiple mutations within a gene
2 can give rise to a similar disease phenotype include
3 osteogenesis imperfecta, familial hypercholesterolemia,
4 retinitis pigmentosa, and many others. The utility of
5 the degeneracy of the genetic code (wobble hypothesis)
6 to enable suppression of one or both alleles of a gene
7 and the introduction of a replacement gene such that
8 it escapes suppression has been exploited in the
9 invention.

10

11 The invention addresses shortcomings of the prior art
12 by providing a novel approach to the design of
13 suppression effectors directed to target alleles of a
14 gene carrying a deleterious mutation. Suppression of
15 every mutation giving rise to a disease phenotype may
16 be costly and problematic. Disease mutations are
17 often single nucleotide changes. As a result
18 differentiating between the disease and normal alleles
19 may be difficult. Some suppression effectors require
20 specific sequence targets, for example, hammerhead
21 ribozymes cleave at NUX sites and hence may not be able
22 to target many mutations. Notably, the wide spectrum
23 of mutations observed in many diseases adds additional
24 complexity to the development of therapeutic strategies
25 for such disorders - some mutations may occur only once
26 in a single patient. A further problem associated with
27 suppression is the high level of homology present in
28 coding sequences between members of some gene families.
29 This can limit the range of target sites for
30 suppression which will enable specific suppression of a
31 single member of such a gene family.

32

33 The present invention circumvents shortcomings in the
34 prior art by utilising the degeneracy of the genetic
35 code. In the invention suppression effectors are
36 designed specifically to sequences in coding regions of

1 genes or in gene products. Typically one allele of the
2 gene contains a mutation with a deleterious effect.
3 Suppression targeted to coding sequences provides
4 greater flexibility in choice of target sequence for
5 suppression in contrast to suppression directed towards
6 single disease mutations. Additionally the invention
7 provides for the introduction of a replacement gene
8 with modified sequences such that the replacement gene
9 is protected from suppression. The replacement gene is
10 modified at third base wobble positions and hence
11 provides the wild type gene product. Notably, the
12 invention has the advantage that the same suppression
13 strategy could be used to suppress, in principle, many
14 mutations in a gene. This is particularly relevant
15 when large numbers of mutations within a single gene
16 cause disease pathology. The replacement gene provides
17 (when necessary) expression of the normal protein
18 product when required to ameliorate pathology
19 associated with reduced levels of wild type protein.
20 The same replacement gene could in principle be used in
21 conjunction with the suppression of many different
22 disease mutations within a given gene. Target
23 sequences may be found in any part of the coding
24 sequence. Suppression in coding sequence holds the
25 advantage that such sequences are present in both
26 precursor and mature RNAs, thereby enabling suppressors
27 to target all forms of RNA.

28
29 In summary the invention involves gene suppression of
30 disease and normal alleles targeting coding sequences
31 in a gene and when necessary gene replacement such
32 that the replacement gene cannot be suppressed.
33 Replacement genes are modified at third base positions
34 (wobble positions) so that they code for the correct
35 amino acids but are protected completely or partially
36 from suppression. The same suppression and replacement

1 steps can be used for many disease mutations in a given
2 gene. Suppression and replacement can be undertaken in
3 conjunction with each other or separately.
4
5

6 DESCRIPTION OF INVENTION
7

8 According to the present invention there is provided a
9 strategy for suppressing expression of an endogenous
10 gene with a deleterious mutation, wherein said strategy
11 comprises providing suppression effectors such as
12 antisense nucleic acids able to bind to sequences of a
13 gene to be suppressed, to prevent the functional
14 expression thereof.
15

16 Generally, the term suppression effectors means nucleic
17 acids, peptide nucleic acids (PNAs), peptides,
18 antibodies or modified forms of these used to silence
19 or reduce gene expression in a sequence specific
20 manner.
21

22 Suppression effectors, such as antisense nucleic acids
23 can be DNA or RNA, can typically be directed to coding
24 sequence however suppression effectors can be targeted
25 to coding sequence and can also be targetted to 5'
26 and/or 3' untranslated regions and/or introns and/or
27 control regions and/or sequences adjacent to a gene or
28 to any combination of such regions of a gene.
29 Antisense nucleic acids including both coding and
30 non-coding sequence can be used if required to help to
31 optimise suppression. Binding of the suppression
32 effector(s) prevents or lowers functional expression of
33 the endogenous gene.
34

35 Generally the term 'functional expression' means the
36 expression of a gene product able to function in a

1 manner equivalent to or better than a wild type
2 product. In the case of a mutant gene or predisposing
3 gene 'functional expression' means the expression of a
4 gene product whose presence gives rise to a deleterious
5 effect or predisposes to a deleterious effect. By
6 deleterious effect is meant giving rise to or
7 predisposing to disease pathology or altering the
8 effect(s) and/or efficiency of an administered
9 compound.

10
11 In a particular embodiment of the invention the
12 strategy further employs ribozymes which can be
13 designed to elicit cleavage of target RNAs. The
14 strategy further employs nucleotides which form triple
15 helix DNA. The strategy can employ peptide nucleic
16 acids for suppression. Nucleic acids for antisense,
17 ribozymes, triple helix^{forming DNA} and peptide nucleic acids may
18 be modified to increase stability, binding efficiencies
19 and uptake (see prior art). Nucleic acids can be
20 incorporated into a vector. Vectors include naked DNA,
21 DNA plasmid vectors, RNA or DNA virus vectors, lipids,
22 polymers or other derivatives and compounds to aid gene
23 delivery and expression.

24
25 The invention further provides the use of antisense
26 nucleotides, ribozymes, PNAs, triple helix nucleotides
27 or other suppression effectors alone or in a vector or
28 vectors, wherein the nucleic acids are able to bind
29 specifically or partially specifically to coding
30 sequences of a gene to prevent or reduce the functional
31 expression thereof, in the preparation of a medicament
32 for the treatment of an autosomal dominant or polygenic
33 disease or to increase the utility and/or action of an
34 administered compound.

35
36 In a further embodiment of the invention target

1 sequences for suppression can include non-coding
2 sequences of the gene.

3
4 According to the present invention there is provided a
5 strategy for suppressing specifically or partially
6 specifically an endogenous gene and (if required)
7 introducing a replacement gene, said strategy
8 comprising the steps of:

- 9
- 10 1. providing nucleic acids or other suppression
11 effectors able to bind to an endogenous gene, gene
12 transcript or gene product to be suppressed and
13
 - 14 2. providing genomic DNA or cDNA (complete or
15 partial) encoding a replacement gene wherein the
16 nucleic acids are unable to bind to equivalent
17 regions in the genomic DNA or cDNA to prevent
18 expression of the replacement gene. The
19 replacement nucleic acids will not be recognised
20 by suppression nucleic acids or will be recognised
21 less effectively than the endogenous gene. The
22 coding sequence of replacement nucleic acids can
23 be altered to prevent or reduce efficiency of
24 suppression. Replacement nucleic acids have
25 modifications in one or more third base (wobble)
26 positions such that replacement nucleic acids
27 still code for the wild type or equivalent amino
28 acids.

29
30 In a particular embodiment of the invention there is
31 provided a strategy for gene suppression targeted to
32 coding sequences of the gene to be suppressed.
33 Suppression will be specific or partially specific to
34 one allele, for example, to the allele carrying a
35 deleterious mutation. Suppressors are targeted to
36 coding regions of a gene or to a combination of coding

1 and non-coding regions of a gene. Suppressors can be
2 targeted to a characteristic of one allele of a gene
3 such that suppression is specific or partially specific
4 to one allele of a gene (PCT/GB97/00574). The
5 invention further provides for use of replacement
6 nucleic acids with altered coding sequences such that
7 replacement nucleic acids will not be recognised (or
8 will be recognised less effectively) by suppression
9 nucleic acids which are targeted specifically or
10 partially specifically to one allele of the gene to be
11 suppressed. Replacement nucleic acids provide the wild
12 type gene product, an equivalent gene product or an
13 improved gene product but are protected completely or
14 partially from suppression effectors targeted to coding
15 sequences.

16
17 In a further embodiment of the invention replacement
18 nucleic acids are provided such that replacement
19 nucleic acids will not be recognised by naturally
20 occurring suppressors found to inhibit or reduce gene
21 expression in one or more individuals, animals or
22 plants. The invention provides for use of replacement
23 nucleic acids which have altered sequences targeted by
24 suppressors of the gene such that suppression by
25 naturally occurring suppressors is completely or
26 partially prevented.

27
28 In an additional embodiment of the invention there is
29 provided replacement nucleic acids with altered
30 nucleotide sequence in coding regions such that
31 replacement nucleic acids code for a product with one
32 or more altered amino acids. Replacement nucleic acids
33 provide a gene product which is equivalent to
34 or improved compared with the naturally occurring
35 endogenous wild type gene product.

36

1 In an additional embodiment of the invention there is
2 provided a strategy to suppress a gene where the gene
3 transcript or gene product interferes with the action
4 of an administered compound.

5
6 The invention further provides the use of a vector or
7 vectors containing suppression effectors in the form of
8 nucleic acids, said nucleic acids being directed
9 towards coding sequences or combinations of coding and
10 non-coding sequences of the target gene and vector(s)
11 containing genomic DNA or cDNA encoding a replacement
12 gene sequence to which nucleic acids for suppression
13 are unable to bind (or bind less efficiently), in the
14 preparation of a combined medicament for the treatment
15 of an autosomal dominant or polygenic disease. Nucleic
16 acids for-suppression or replacement gene nucleic acids
17 may be provided in the same vector or in separate
18 vectors. Nucleic acids for suppression or replacement
19 gene nucleic acids may be provided as a combination of
20 nucleic acids alone or in vectors.

21
22 The invention further provides a method of treatment
23 for a disease caused by an endogenous mutant gene, said
24 method comprising sequential or concomitant
25 introduction of

26
27 (a) nucleic acids to the coding regions of a gene to
28 be suppressed and/or nucleic acids to coding
29 regions and any combination of 5' and/or 3'
30 untranslated regions, intronic regions, control
31 regions or regions adjacent to a gene to be
32 suppressed

33
34 (b) replacement nucleic acids with sequences which
35 allow the replacement gene to be expressed.

36

1 The nucleic acid for gene suppression can be
2 administered before, after or at the same time as the
3 replacement gene is administered.

4
5 The invention further provides a kit for use in the
6 treatment of a disease caused by a deleterious mutation
7 in a gene, the kit comprising nucleic acids for
8 suppression able to bind to the gene to be suppressed
9 and if required a replacement nucleic acid to replace
10 the mutant gene having sequence which allows it to be
11 expressed and completely or partially escape
12 suppression.

13
14 Nucleotides can be administered as naked DNA or RNA.
15 Nucleotides can be delivered in vectors. Naked nucleic
16 acids or nucleic acids in vectors can be delivered with
17 lipids or other derivatives which aid gene delivery.
18 Nucleotides may be modified to render them more stable,
19 for example, resistant to cellular nucleases while
20 still supporting RNaseH mediated degradation of RNA or
21 with increased binding efficiencies (see prior art).
22 Antibodies or peptides can be generated to target the
23 protein product from the gene to be suppressed.

24
25 The strategy described herein has applications for
26 alleviating autosomal dominant diseases. Complete
27 silencing of a disease allele may be difficult to
28 achieve using antisense, PNA, ribozyme and triple helix
29 approaches or any combination of gene silencing
30 approaches. However small quantities of mutant product
31 may be tolerated in some autosomal dominant disorders.
32 In others a significant reduction in the proportion of
33 mutant to normal product may result in an amelioration
34 of disease symptoms. Hence this invention may be
35 applied to any autosomal dominantly or polygenically
36 inherited disease in man where the molecular basis of

1 the disease has been established or is partially
2 understood. This strategy will enable the same therapy
3 to be used to treat a range of different disease
4 mutations within the same gene. The development of
5 such approaches will be important to future therapies
6 for autosomal dominant and polygenic diseases, the key
7 to a general strategy being that it circumvents the
8 need for a specific therapy for every mutation causing
9 or predisposing to a disease. This is particularly
10 relevant in some disorders, for example, rhodopsin
11 linked autosomal dominant RP, in which to date about
12 one hundred different mutations in the rhodopsin gene
13 have been observed in adRP patients. Likewise hundreds
14 of mutations have been identified in the human type I
15 Collagen 1A1 and 1A2 genes in autosomal dominant
16 osteogenesis imperfecta. Costs of developing therapies
17 for each mutation are prohibitive at present.
18 Inventions such as this using a general approach for
19 therapy will be required. General approaches may be
20 targeted to the primary defect as is the case with this
21 invention or to secondary effects such as apoptosis.

22
23 This invention may be applied in gene therapy
24 approaches for biologically important polygenic
25 disorders affecting large proportions of the world's
26 populations such as age related macular degeneration,
27 glaucoma, manic depression, cancers having a familial
28 component and indeed many others. Polygenic diseases
29 require inheritance of more than one mutation
30 (component) to give rise to the disease state. Notably
31 an amelioration in disease symptoms may require
32 reduction in the presence of only one of these
33 components, that is, suppression of one genotype which,
34 together with others leads to the disease phenotype,
35 may be sufficient to prevent or ameliorate symptoms of
36 the disease. In some cases suppression of more than

1 one component may be required to improve disease
2 symptoms. This invention may be applied in possible
3 future interventive therapies for common polygenic
4 diseases to suppress a particular genotype(s) using
5 suppression and when necessary replacement steps.

6
7 The present invention is exemplified using four genes:
8 human rhodopsin, mouse rhodopsin, human peripherin and
9 human collagen 1A2. The first of these genes are
10 retinal specific. In contrast, collagen 1A2 is
11 expressed in a range of tissues including skin and
12 bone. While these four genes have been used as
13 examples there is no reason why the invention could not
14 be deployed in the suppression of many other genes in
15 which mutations cause or predispose to a deleterious
16 effect. Many examples of mutant genes which give rise
17 to disease phenotypes are available from the prior art
18 - these genes all represent targets for the invention.
19 The present invention is exemplified using hammerhead
20 ribozymes with antisense arms to elicit RNA cleavage.
21 There is no reason why other suppression effectors
22 directed towards genes, gene transcripts or gene
23 products could not be used to achieve gene suppression.
24 Many examples from the prior art detailing use of
25 suppression effectors inter alia antisense RNA/DNA,
26 triple helix, ^{forming DNA} PNAS and peptides to achieve suppression
27 of gene expression are reported (see prior art).
28 The present invention is exemplified using hammerhead
29 ribozymes with antisense arms to elicit sequence
30 specific cleavage of transcripts from genes implicated
31 in dominant disorders and non-cleavage of transcripts
32 from replacement genes containing sequence
33 modifications in wobble positions such that the
34 replacement gene still codes for wild type protein.
35 The present invention is exemplified using suppression
36 effectors targeting sites in coding regions of the

1 human and mouse rhodopsin, human peripherin and human
2 collagen 1A2 genes. Target sites which include
3 sequences from transcribed but untranslated regions of
4 genes, introns, regions involved in the control of gene
5 expression, regions adjacent to the gene or any
6 combination of these could be used to achieve gene
7 suppression. Multiple suppression effectors, for
8 example, shotgun ribozymes could be used to optimise
9 efficiency of suppression when necessary.
10 Additionally, when required expression of a modified
11 replacement gene such that the replacement gene product
12 is altered from the wild type product such that it
13 provides a beneficial effect may be used to provide
14 gene product.

15

16

17 MATERIALS and METHODS

18

19

20 Cloning vectors

21

22 cDNA templates and ribozymes were cloned into
23 commercial expression vectors (pCDNA3, pZeoSV or
24 pBluescript) which enable expression in a test tube
25 from T7, T3 or SP6 promoters or expression in mammalian
26 cells from CMV or SV40 promoters. [Inserts were placed] DNA inserts were
27 into the multiple cloning site (MCS) of these vectors cloned
28 typically at or near the terminal ends of the MCS to
29 delete most of the MCS and thereby prevent any possible
30 problems with efficiency of expression subsequent to
31 cloning.

32

33 Sequencing protocols

34

35 Clones containing template cDNAs and ribozymes were
36 sequenced by ABI automated sequencing machinery using

1 standard protocols.

2

3 Expression of RNAs

4

5 RNA was obtained from clones ^{by} *in vitro* ^{transcription} using a
6 commercially available Ribomax expression system
7 (Promega) and standard protocols. RNA purifications
8 were undertaken using the Bio-101 RNA purification kit
9 or a solution of 0.3M sodium acetate and 0.2% SDS after
10 isolation from polyacrylamide gels. Cleavage reactions
11 were performed using standard protocols with varying
12 MgCl₂ concentrations (0-15mM) at 37°C, typically for 3
13 hours. Time points were performed at the predetermined
14 optimal MgCl₂ concentrations for up to 5 hours.
15 Radioactively labelled RNA products were obtained by
16 incorporating a-P₃₂ ^{subscript} rUTP (Amersham) in the expression
17 reactions (Gaughan et al. 1995). Labelled RNA products
18 were run on polyacrylamide gels before cleavage
19 reactions were undertaken for the purpose of RNA
20 purification and subsequent to cleavage reactions to
21 establish if RNA cleavage had been achieved. Cleavage
22 reactions were undertaken with 5mM Tris-HCl pH8.0 and
23 varying concentrations of MgCl₂ at 37°C.

24

25 RNA secondary structures

26

27 Predictions of the secondary structures of human and
28 mouse rhodopsin, human peripherin and human collagen
29 1A2 mRNAs were obtained using the RNAPlotFold program.
30 Ribozymes and antisense were designed to target areas
31 of the RNA that were predicted to be accessible to
32 suppression effectors, for example open loop
33 structures. The integrity of open loop structures was
34 evaluated from the 10 most probable RNA structures.
35 Additionally, predicted RNA structures for truncated
36 RNA products were generated and the integrity of open

1 loops between full length and truncated RNAs compared.

2

3 TEMPLATES and RIBOZYMES

4

5 Human Rhodopsin

6

7 Template cDNA

8 The human rhodopsin cDNA was cloned into the HindIII
9 and EcoRI sites of the MCS of pCDNA3 in a 5' to 3'
10 orientation allowing subsequent expression of RNA from
11 the T7 or CMV promoter in the vector. The full length
12 5'UTR sequence was inserted into this clone using
13 primer driven PCR mutagenesis and a HindIII (in pCDNA3)
14 to BstEII (in the coding sequence of the human
15 rhodopsin cDNA) DNA fragment [Sequence 1].

16

17 cDNA with altered sequence at a wobble position

18 The human rhodopsin hybrid cDNA with a single base
19 alteration, a C-->G change (at ^{nucleotide 271 of SEQ ID NO:2} ~~position 477~~) was

20 introduced into human rhodopsin cDNA, using a HindIII
21 to BstEII PCR cassette, by primer directed PCR
22 mutagenesis. This sequence change occurs at a silent
23 position - it does not give rise to an amino acid
24 substitution - however it eliminates the ribozyme
25 cleavage site (GUX -->GUG). The hybrid rhodopsin was
26 cloned into pCDNA3 in a 5' to 3' orientation allowing
27 subsequent expression of RNA from the T7 or CMV
28 promoter in the vector [Sequence 2].

29

30 Rhodopsin cDNA carrying a Pro23Leu adRP mutation

31 A human rhodopsin adRP mutation, a C-->T change (at
32 codon 23) was introduced into human rhodopsin cDNA,
33 using a HindIII to BstEII PCR cassette by primer
34 directed PCR mutagenesis. This sequence change results
35 in the substitution of a Proline for a Leucine residue.
36 Additionally the nucleotide change creates a ribozyme

(nucleotide 217 of
SEQ ID NO:3)

1 cleavage site (CCC-->CTC). The mutated rhodopsin was
 2 cloned into the HindIII and EcoRI sites of pCDNA3 in a
 3 5' to 3' orientation allowing subsequent expression of
 4 RNA from the T7 or CMV promoter in the vector
 5 (Sequence 3).
 6 *SEE ID NO:*

7 Ribozyme constructs

8 A hammerhead ribozyme (termed Rz10) designed to target
 9 a large conserved open loop structure in the RNA from
 10 the coding regions of the gene was cloned subsequent to
 11 synthesis and annealing into the HindIII and XbaI sites
 12 of pCDNA3 again allowing expression of RNA from the T7
 13 or CMV promoter in the vector (Sequence 4). The target
 14 site was GUC (the GUX rule) at position 475-477 of the
 15 human rhodopsin sequence. A hammerhead ribozyme

16 (termed Rz20) designed to target an open loop structure
 17 in RNA from the coding region of a mutant rhodopsin
 18 gene with a Pro23Leu mutation was cloned subsequent to
 19 synthesis and annealing into the HindIII and XbaI sites
 20 of pCDNA3 again allowing expression of RNA from the T7
 21 or CMV promoter in the vector (Sequence 5). The target
 22 site was CTC (the NUX rule) at codon 23 of the human
 23 rhodopsin sequence (Accession number: K02281).

24 Antisense flanks are underlined.

25 Rz10: GGACGGTCTGATGAGTCCGTGAGGACGAAACGTAGAG

26 Rz20: TACTCGAACTGATGAGTCCGTGAGGACGAAAGGCTGC

27 *positions 35 and 37 are g's.*

28 Mouse rhodopsin

29

30 Template cDNA

31 The full length mouse rhodopsin cDNA was cloned into
 32 the EcoRI sites of the MCS of pCDNA3 in a 5' to 3'
 33 orientation allowing subsequent expression of RNA from
 34 the T7 or CMV promoter in the vector (Sequence 6).
 35 *SEE ID NO:*

36 cDNA with altered sequence at a wobble position

nucleotide 216-218 SEE ID NO: 6

nucleic acid sequence

(SEE ID NO: 4)

(nucleotide 369-371 of SEE ID NO: 1)

(nuc. 216-218 of SEE ID NO: 3)

(nuc. 101-137 of SEE ID NO: 4)

(nuc. 104-140 of SEE ID NO: 5)

1 The mouse rhodopsin hybrid cDNA with a single base
 2 alteration, a T-->C change (at position 1460) was
 3 introduced into mouse rhodopsin cDNA, using a HindIII
 4 to Eco47III PCR cassette, by primer directed PCR
 5 mutagenesis. This sequence change occurs at a silent
 6 position - it does not give rise to an amino acid
 7 substitution - however it eliminates the ribozyme
 8 cleavage site (TTT-->TCT). The hybrid rhodopsin was
 9 cloned into pCDNA3 in a 5' to 3' orientation allowing
 10 subsequent expression of RNA from the T7 or CMV
 11 promoter in the vector (Sequence 7).
 12

nuc. 190 of
 SEQ ID NO. 7

13 Ribozyme constructs

14 A hammerhead ribozyme (termed Rz33) designed to target
 15 a large robust open loop structure in the RNA from the
 16 coding regions of the gene was cloned subsequent
 17 to synthesis and annealing into the HindIII and XbaI
 18 sites of pCDNA3 again allowing expression of RNA from
 19 the T7 or CMV promoter in the vector (Sequence 8).
 20 The target site was TTT (the NUX rule) at position
 21 1459-1461 of the mouse rhodopsin sequence. (Accession
 22 number: M55171). Antisense flanks are underlined.

SEQ ID NO. 7
 nuc. 189-191 of SEQ ID NO. 7

23 Rz33: GGCACATCTGATGAGTCCGTGAGGACGAAAAAATTGG (Sequence 8)

nuc. 118-154 of

24 (nuc. 405-407 of SEQ ID NO. 3)

25 Human peripherin

27 Template cDNA

28 The full length human peripherin cDNA was cloned into
 29 the EcoRI sites of the MCS of pCDNA3 in a 5' to 3'
 30 orientation allowing subsequent expression of RNA from
 31 the T7 or CMV promoter in the vector (Sequence 9).
 32

33 DNAs with altered sequence at a wobble position

34 A human peripherin hybrid DNA with a single base
 35 alteration, a A-->G change (at position 257) was
 36 introduced into human peripherin DNA by primer directed

(nuc. 332 of SEQ ID NO. 10)

1 PCR mutagenesis (forward) 257 mutation primer -
 2 5'CATGGCGCTGCTGAAAGTCA3' - the reverse 257 primer was
 3 complementary to the forward primer). This sequence
 4 change occurs at a silent position - it does not give
 5 rise to an amino acid substitution - however it
 6 eliminates the ribozyme cleavage site
 7 (CTA-->CTG) (Sequence 10). A second human peripherin
 8 hybrid DNA with a single base alteration, a A-->G
 9 change (at position 359) was introduced into human
 10 peripherin DNA, again by primer directed PCR
 11 mutagenesis (forward 359 mutation primer -
 12 5'CATCTTCAGCCTGGGACTGT3' - the reverse 359 primer was
 13 complementary to the forward primer) (Sequence 11).
 14 Similarly this sequence change occurs at a silent
 15 position - it does not give rise to an amino acid
 16 substitution - however again it eliminates the ribozyme
 17 cleavage site (CTA-->CTG). The ribozyme cleavage sites
 18 at 255-257 and 357-359 occur at different open loop
 19 structures as predicted by the RNAPlotFold program.
 20 Hybrid peripherin DNAs included the T7 promoter
 21 sequence allowing subsequent expression of RNA.
 22
 23 Ribozyme constructs
 24 Hammerhead ribozymes (termed Rz30 and Rz31), designed
 25 to target robust open loop structures in the RNA from
 26 the coding regions of the gene, were cloned subsequent
 27 to synthesis and annealing into the HindIII and XbaI
 28 sites of pCDNA3 again allowing expression of RNA from
 29 the T7 or CMV promoter in the vector (Sequences 14 and 15
 30 12+13). The target sites were both CTA (the NUX rule) respectively
 31 at positions 255-257 and 357-359 respectively of the
 32 human peripherin sequence. (Accession number: M73531).
 33 Antisense flanks are underlined.
 34 Rz30: ACTTTCAGCTGATGAGTCCGTGAGGACGAAAGCGCCA (nucleotide 116-153 of SEQ ID No: 14)
 35 Rz31: ACAGTCCCTGATGAGTCCGTGAGGACGAAAGGCTGAA (nucleotide 112-148 of SEQ ID No: 15)
 36

(nuc. 330-352
 of SEQ ID NO. 10)

(SEQ ID NO. 11)

nuc. 468 of SEQ
 ID NO. 13

(SEQ ID NO. 12)

(SEQ ID NO. 13)

(nuc. 466-468 of SEQ ID NO. 13)

(SEQ ID NOs: 14 and 15)

1 Human Type I Collagen - COL1A2

2

3 Template cDNA

4 A human type I collagen 1A2 cDNA was obtained from the
 5 ATCC (Accession No: Y00724). A naturally occurring
 6 polymorphism has previously been found in collagen 1A2
 7 at positions 907 of the gene involving a T-->A
 8 nucleotide change (Filie et al. 1993). The
 9 polymorphism occurs in a predicted open loop structure
 10 of human collagen 1A2 RNA. Polymorphic variants of
 11 human collagen 1A2 were generated by PCR directed
 12 mutagenesis, using a HindIII to XbaI PCR cassette.

13 Resulting clones contained the following polymorphism : (nuc. 181 of
 14 collagen 1A2 (A) has a A nucleotide at position 907 → SEQ ID NO: 16).
 15 [~~Sequence 14~~] In contrast human collagen 1A2 (B) has
 16 a T nucleotide at position 907 (Sequence 15). In
 17 collagen 1A2 (B) there is a ribozyme target site, that
 18 is a GTC site (906-908), however this cleavage site is
 19 lost in collagen 1A2 (A) as the sequence is altered to
 20 GAC (906-908), thereby disrupting the ribozyme target
 21 site. (nuc. 174-175 of SEQ ID NO: 17)
 22 (nuc. 180-182 of SEQ ID NO: 16)

23 Ribozyme constructs

24 A hammerhead ribozyme (termed Rz907) was designed to
 25 target a predicted open loop structure in the RNA from
 26 the coding region of the polymorphic variant of the
 27 human collagen 1A2 gene. Rz907 oligonucleotide primers
 28 were synthesised, annealed and cloned into the HindIII
 29 and XbaI sites of pCDNA3 again allowing subsequent
 30 expression of RNA from the T7 or CMV promoter in the
 31 vector (Sequence 16). The target site was a GUX site
 32 at position 906-908 of the human type I collagen 1A2
 33 sequence (Accession number: Y00724). Antisense flanks
 34 are underlined.

35 Rz907: CGGCGGCTGATGAGTCCGTGAGGACGAAACCAGCA (nuc. 107-141 of SEQ ID NO.
 36 18)

FIGURE LEGENDS

Diagram 1

pBR322 was cut with MspI, radioactively labeled and run on a polyacrylamide gel to enable separation of the resulting DNA fragments. The sizes of these fragments are given in diagram 1. This DNA ladder was then used on subsequent polyacrylamide gels (4-8%) to provide an estimate of the size of the RNA products run on the gels. However there is a significant difference in mobility between DNA and RNA depending on the percentage of polyacrylamide and the gel running conditions - hence the marker provides an estimate of size of transcripts.

Figure 1 -

A: Human rhodopsin cDNA^(SEQ ID NO: 1) was expressed from the T7 promoter to the BstEII site in the coding sequence. Resulting RNA was mixed with R210RNA in 15mM magnesium chloride and incubated at 37°C for varying times. Lanes 1-4: Human rhodopsin RNA and R210RNA after incubation at 37°C with 15mM magnesium chloride for 0, 1 2 and 3 hours respectively. Sizes of the expressed RNAs and cleavage products are as expected (Table 1). Complete cleavage of human rhodopsin RNA was obtained with a small residual amount of intact RNA present at 1 hour. Lane 6 is intact unadapted human rhodopsin RNA (BstEII) alone. Lane 5 is unadapted human rhodopsin RNA (FspI) alone and refers to Figure 1B. From top to bottom, human rhodopsin RNA and the two cleavage products from this RNA are highlighted with arrows.

B: The unadapted human rhodopsin cDNA was expressed from the T7 promoter to the FspI site in the coding sequence. The adapted human rhodopsin cDNA was expressed from the T7 promoter to the BstEII site in the coding sequence. Lanes 1-4: Resulting RNAs were

1 mixed together with Rz10 and 15mM magnesium chloride
2 and incubated at 37°C for varying times (0, 1, 2 and 3
3 hours respectively). The smaller unadapted rhodopsin
4 transcripts were cleaved by Rz10 while the larger
5 adapted transcripts were protected from cleavage by
6 Rz10. Cleavage of adapted protected transcripts would
7 have resulted in products of 564bases and 287bases -
8 the 564bases product clearly is not present - the 287bp
9 product is also generated by cleavage of the unadapted
10 human rhodopsin transcripts and hence is present (FspI).
11 After 3 hours the majority of the unadapted rhodopsin
12 transcripts has been cleaved by Rz10. Lane 5 contains
13 the intact adapted human rhodopsin RNA (BstEII) alone.
14 From top to bottom adapted uncleaved human rhodopsin
15 transcripts, residual unadapted uncleaved human
16 rhodopsin transcripts and the larger of the cleavage
17 products from unadapted human rhodopsin transcripts are
18 highlighted by arrows. The smaller 22bases cleavage
19 product from the unadapted human rhodopsin transcripts
20 has run off the gel.
21

22 Figure 2

23 A: Unadapted and adapted human rhodopsin cDNAs were
24 expressed from the T7 promoter to the AcyI after the
25 coding sequence and the BstEII site in the coding
26 sequence respectively. Sizes of expressed RNAs and
27 cleavage products were as predicted (Table 1).
28 Resulting RNAs were mixed together with Rz10RNA at
29 varying magnesium chloride concentrations and incubated
30 at 37°C for 3 hours. Lane 1: Intact unadapted human
31 rhodopsin RNA (AcyI) alone. Lanes 2-5: Unadapted and
32 adapted human rhodopsin RNAs and Rz10RNA after
33 incubation at 37°C with 0, 5, 10 and 15 mM MgCl₂
34 respectively. Almost complete cleavage of the larger
35 unadapted human rhodopsin RNA was obtained with a small
36 residual amount of intact RNA present at 5 mM MgCl₂. In

1 contrast the adapted human rhodopsin RNA remained
2 intact. From top to bottom, the unadapted and adapted
3 rhodopsin RNAs, and two cleavage products from the
4 unadapted human rhodopsin RNA are highlighted by
5 arrows. Lane 6 is intact adapted human rhodopsin RNA
6 (BstEII) alone.

7 B: The adapted human rhodopsin cDNA was expressed from
8 the T7 promoter to the BstEII site in the coding
9 sequence. Lanes 1-4: Resulting RNA was mixed together
10 with Rz10 and 0, 5, 10 and 15 mM magnesium chloride
11 and incubated at 37°C for 3 hours respectively. The
12 adapted rhodopsin transcripts were not cleaved by Rz10.
13 Cleavage of adapted transcripts would have resulted in
14 cleavage products of 564~~bases~~ and 287~~bases~~ which
15 clearly are not present. Lane 5: intact adapted human
16 rhodopsin RNA (BstEII) alone. Lane 4: RNA is absent -
17 due to a loading error or degradation. The adapted
18 uncleaved human rhodopsin RNA is highlighted by an
19 arrow. (564 bp) (287 bp)

20 C: Unadapted and adapted human rhodopsin cDNAs were
21 expressed from the T7 promoter to the AcyI after the
22 coding sequence and the BstEII site in the coding
23 sequence respectively. Sizes of expressed RNAs and
24 cleavage products were as predicted (Table 1).
25 Resulting RNAs were mixed together with Rz10RNA at
26 varying magnesium chloride concentrations and
27 incubated at 37°C for 3 hours. Lane 1: DNA ladder as
28 in Diagram 1. Lanes 2-5: Unadapted and adapted human
29 rhodopsin RNAs and Rz10RNA after incubation at 37°C
30 with 0, 5, 10 and 15 mM MgCl₂ respectively. Almost
31 complete cleavage of the larger unadapted human
32 rhodopsin RNA was obtained with a small residual amount
33 of intact RNA present at 5 and 10 mM MgCl₂. In contrast
34 the adapted human rhodopsin RNA remained intact. Lane
35 6: Adapted human rhodopsin RNA (BstEII) alone. Lane 7:
36 Unadapted human rhodopsin RNA (AcyI) alone. Lane 8:

1 DNA ladder as in Diagram 1. From top to bottom, the
2 unadapted and adapted rhodopsin RNAs, and two cleavage
3 products from the unadapted human rhodopsin RNA are
4 highlighted by arrows. Separation of the adapted human
5 rhodopsin RNA (851bases) and the larger of the cleavage
6 products from the unadapted RNA (896bases) is
7 incomplete in this gel (further runnning of the gel
8 would be required to achieve separation) - however the
9 separation of these two RNAs is demonstrated in Figure
10 2A.

11
12 Figure 3

13 The mutant (Pro23Leu) ^(see ID NO. 3) human rhodopsin cDNA was
14 expressed from the T7 promoter to the BstEII in the
15 coding sequence. Likewise the Rz20 clone was expressed
16 to the XbaI site. Resulting RNAs were mixed together
17 with 10mM magnesium chloride concentrations at 37°C for
18 varying times. Sizes of expressed RNAs and cleavage
19 products were as predicted (Table 1). Lane 1: DNA
20 ladder as in Diagram 1. Lanes 2: Pro23Leu human
21 rhodopsin RNA alone. Lanes 3-7 Pro23Leu human
22 rhodopsin RNA and Rz20 RNA after incubation at 37°C with
23 10 mM MgCl₂ for 0mins, 30 mins, 1 hr, 2hrs and 5hrs
24 respectively. Almost complete cleavage of mutant
25 rhodopsin transcripts was obtained with a residual
26 amount of intact RNA left even after 5 hours. Lane
27 8: DNA ladder as in Diagram 1. From top to bottom,
28 intact mutant rhodopsin RNA and the two cleavage
29 products from the mutant human rhodopsin RNA are
30 highlighted by arrows.

31
32 Figure 4

33 The mutant (Pro23Leu) ^(see ID NO. 3) human rhodopsin cDNA was
34 expressed from the T7 promoter to the BstEII in the
35 coding sequence. Likewise the Rz10 clone was expressed
36 to the XbaI site. Resulting RNAs were mixed together

(see ID NO. 4)

1 with 10mM magnesium chloride concentrations at 37°C for
2 varying times. Sizes of expressed RNAs and cleavage
3 products were as predicted (Table 1). Lane 1: DNA
4 ladder as in Diagram 1. Lanes 2: Pro23Leu human
5 rhodopsin RNA alone. Lanes 3-7 Pro23Leu human
6 rhodopsin RNA and Rz10 RNA after incubation at 37°C with
7 10 mM MgCl₂ for 0mins, 30 mins, 1 hr, 2hrs and 5hrs
8 respectively. Almost complete cleavage of mutant human
9 rhodopsin RNA was obtained with a residual amount of
10 intact RNA remaining even after 5 hours (Lane 7). Lane
11 8: DNA ladder as in Diagram 1. From top to bottom,
12 intact mutant rhodopsin RNA and the two cleavage
13 products from the mutant human rhodopsin RNA
14 are highlighted by arrows.

15

16 Figure 5 -

17 The mouse rhodopsin cDNA clone was expressed in vitro
18 from the T7 promoter to the Eco47III site in the coding
19 sequence. Likewise the Rz33 clone was expressed to the
20 XbaI site. A: Resulting RNAs were mixed together with
21 10mM magnesium chloride at 37°C for varying times.
22 Sizes of expressed RNAs and cleavage products were as
23 predicted (Table 1). DNA ladder as in Diagram 1. Lane
24 1: mouse rhodopsin RNA alone. Lanes 2-5 Mouse
25 rhodopsin RNA and Rz33 RNA after incubation at 37°C
26 with 10 mM MgCl₂ at 0, 5, 7.5 and 10 mM MgCl₂
27 respectively for 3 hours. Cleavage of mouse rhodopsin
28 RNA was obtained after addition of divalent ions (Lane
29 3). Residual uncleaved mouse rhodopsin RNA and the two
30 cleavage products from the mouse rhodopsin RNA are
31 highlighted by arrows. B: The adapted mouse rhodopsin
32 cDNA clone with a base change at (position 1460) was
33 expressed in vitro from the T7 promoter to the Eco47III
34 site in the coding sequence. Likewise the Rz33 clone
35 was expressed to the XbaI site. Resulting RNAs were
36 mixed together with various magnesium chloride

(nuc. 190 of
SEQ ID No: 7)

1 concentrations at 37°C for 3 hours. Sizes of expressed
2 RNAs and cleavage products were as predicted (Table 1).
3 Lane 1: DNA ladder as in Diagram 1. Lane 2: Adapted
4 mouse rhodopsin RNA alone. Lanes 3-6: Adapted mouse
5 rhodopsin RNA and Rz33 RNA after incubation at 37°C
6 with 0, 5, 7.5 and 10 mM $MgCl_2$ for 3 hours at 37°C. No
7 cleavage of the adapted mouse rhodopsin RNA was
8 observed. The intact adapted mouse rhodopsin RNA is
9 highlighted by an arrow.

10

11 Figure 6

12 The human peripherin cDNA clone was expressed in vitro
13 from the T7 promoter to the BglIII site in the coding
14 sequence. Likewise Rz30 (targeting a cleavage site
15 at 255-257) was expressed to the XbaI site. A:
16 Resulting RNAs were mixed together with 10mM magnesium
17 chloride at 37°C for varying times. Lane 1: DNA ladder
18 as in Diagram 1. Lane 2: Intact human peripherin RNA
19 alone. Lanes 3-7: Human peripherin RNA and Rz30 RNA
20 after incubation at 37°C with 10 mM $MgCl_2$ for 3hrs,
21 2hrs, 1hr, 30 mins and 0 mins respectively. Lane 8:
22 DNA ladder as in Diagram 1. From top to bottom, intact
23 human peripherin RNA and the two cleavage products from
24 the human peripherin RNA are highlighted by arrows. B:
25 Resulting RNAs were mixed with Rz30 RNA at varying
26 magnesium chloride concentrations and incubated at 37°C
27 for 3hrs. Lane 1: DNA ladder as in Diagram 1. Lanes
28 2-5: Human peripherin RNA and Rz30 after incubation at
29 37°C with 10, 7.5, 5 and 0 mM magnesium chloride
30 respectively for 3hrs. Lane 6: Intact human peripherin
31 RNA alone. Sizes of the expressed RNAs and cleavage
32 products are as expected (Table 1). Significant
33 cleavage of human peripherin RNA was obtained with a
34 residual amount of intact RNA present at each $MgCl_2$
35 concentration. From top to bottom, human peripherin
36 RNA and the two cleavage products from this RNA are

1 highlighted with arrows. C: The adapted human
2 peripherin DNA with a single base change at position
3 257 was expressed from the T7 promoter to the AvrII
4 site in the coding sequence. Resulting RNA was mixed
5 with Rz30 at various magnesium chloride concentrations
6 and incubated at 37°C for 3hrs. Lane 1: DNA ladder as
7 in Diagram 1. Lane 2: Intact adapted human peripherin
8 RNA alone. Lanes 3-6: Adapted human peripherin RNA and
9 Rz30 after incubation at 37°C with 0, 5, 7.5 and 10 mM
10 magnesium chloride respectively for 3hrs. Lane 7: DNA
11 ladder as in Diagram 1. D: The unadapted human
12 peripherin cDNA and the adapted human peripherin DNA
13 fragment with a single base change at position 257 were
14 expressed from the T7 promoter to the BglII and AvrII
15 sites respectively in the coding sequence. Resulting
16 RNAs were mixed with Rz30 at various magnesium chloride
17 concentrations and incubated at 37°C for 3hrs. Lane 1:
18 DNA ladder as in Diagram 1. Lane 2: Intact unadapted
19 human peripherin RNA alone. Lane 3: Intact adapted
20 human peripherin RNA alone. Lanes 4-7: Unadapted and
21 adapted human peripherin RNAs and Rz30 after incubation
22 at 37°C with 0, 5, 7.5 and 10 mM magnesium chloride
23 respectively for 3hrs at 37°C. No cleavage of the
24 adapted human peripherin RNA was observed even after 3
25 hours whereas a significant reduction in the unadapted
26 RNA was observed over the same time frame. Lane 8: DNA
27 ladder as in Diagram 1. From top to bottom, residual
28 unadapted human peripherin RNA, adapted human
29 peripherin RNA and the two cleavage products are
30 highlighted by arrows.

31

32 Figure 7

33 Human peripherin cDNA clone was expressed in vitro from
34 the T7 promoter to the BglII site in the coding
35 sequence. Likewise the Rz31 (targeting a cleavage site
36 at 357-359)_A was expressed to the XbaI site. A:

→ nucleotides
(466-4680) SEB IDNO.13)

1 Resulting RNAs were mixed together with 10mM magnesium
2 chloride at 37°C for varying times. Lane 1: DNA ladder
3 as in Diagram 1. Lanes 2-6: Human peripherin RNA and
4 Rz31 RNA after incubation at 37°C with 10mM MgCl₂ for
5 3hrs, 2hrs, 1hr, 30mins and 0mins respectively.
6 Increased cleavage of mouse rhodopsin RNA was obtained
7 over time - however significant residual intact RNA
8 remained even after 3 hours (Lane 2). Lane 7: Intact
9 human peripherin RNA alone. Lane 8: DNA ladder as in
10 Diagram 1. From top to bottom, intact human peripherin
11 RNA and the two cleavage products from the human
12 peripherin RNA are highlighted by arrows. B: Resulting
13 RNAs were mixed with Rz31 RNA at varying magnesium
14 chloride concentrations and incubated at 37°C for 3hrs.
15 Lane 1: DNA ladder as in Diagram 1. Lanes 2-5: Human
16 peripherin RNA and Rz31 after incubation at 37°C with
17 10, 7.5, 5 and 0m M magnesium chloride respectively for
18 3hrs. Sizes of the expressed RNAs and cleavage
19 products are as expected (Table 1). Significant
20 cleavage of human peripherin RNA was obtained with a
21 residual amount of intact RNA present at each MgCl₂
22 concentration (Lanes 2-4). Lane 6: Intact human
23 peripherin RNA alone. Lane 7: DNA ladder as in Diagram
24 1. From top to bottom, human peripherin RNA and the
25 two cleavage products from this RNA are highlighted
26 with arrows. C: The adapted human peripherin DNA with
27 a single base change at (position 359) was expressed from
28 the T7 promoter to the BglII site in the coding (nucl. 46808 SE0
29 sequence. Resulting RNA was mixed with Rz31 at various 12NO13)
30 magnesium chloride concentrations and incubated at 37°C
31 for 3hrs. Lane 1: DNA ladder as in Diagram 1. Lane 2:
32 Intact adapted human peripherin RNA alone. Lanes 3-6:
33 Adapted human peripherin RNA and RZ31 after incubation
34 at 37°C with 0, 5, 7.5 and 10mM magnesium chloride
35 respectively for 3hrs. No cleavage of the adapted
36 human peripherin RNA was observed even after 3 hours.

1 Lane 7: DNA ladder as in Diagram 1.

2

3 Figure 8

4 A: The human collagen 1A2 cDNA clones containing the A
5 and T alleles of the polymorphism at position 907 were
6 expressed from the T7 promoter to the MvnI and XbaI
7 sites in the insert and vector respectively. Resulting
8 RNAs were mixed together with Rz907 and various MgCl₂
9 concentrations and incubated at 37°C for 3 hours. Lane
10 1: intact RNA from the human collagen 1A2 (A)
11 containing the A allele of the 907 polymorphism. Lane
12 2: intact RNA from the human collagen 1A2 (B)
13 containing the T allele of the 907 polymorphism. Lanes
14 3-5: Human collagen 1A2 (A) and (B) representing the A
15 and T allele RNAs and Rz907 incubated with 0, 5, and 10
16 mM MgCl₂ at 37°C for 3 hours. RNA transcripts from the
17 T allele containing the 906-908 target site are cleaved
18 by Rz907 upon addition of divalent ions - almost
19 complete cleavage is obtained with a residual amount of
20 transcript from the T allele remaining (Lane 5). In
21 contrast transcripts expressed from the A allele (which
22 are smaller in size to distinguish between the A (MvnI)
23 and T (XbaI) alleles) were not cleaved by Rz907 - no
24 cleavage products were observed. From top to bottom,
25 RNA from the T allele, the A allele and the two
26 cleavage products from the T allele are highlighted by
27 arrows. Lane 6: DNA ladder as in Diagram 1.

28 B: The human collagen 1A2 cDNA (A) + (B) clones
29 containing the A and T alleles of the polymorphism at
30 907 were expressed from the T7 promoter to the MvnI and
31 XbaI sites in the insert and vector respectively.
32 Resulting RNAs were mixed together with Rz907 and 10mM
33 magnesium chloride and incubated at 37°C for varying
34 times. Lane 1: DNA ladder as in Diagram 1. Lane 2:
35 intact RNA from the human collagen 1A2 (A) with the A
36 allele of the 907 polymorphism. Lane 3: intact RNA

1 from the human collagen 1A2 (B) with the T allele of
2 the 907 polymorphism. Lanes 4-9: Human collagen 1A2 A
3 and T allele RNA and Rz907 incubated with 10mM MgCl₂ at
4 37°C for 0, 30 mins, 1 hour, 2 hours, 3 hours and
5 5 hours respectively. RNA transcripts from the T allele
6 containing the 906-908 target site are cleaved by Rz907
7 - almost complete cleavage is obtained after 5 hours.
8 In contrast transcripts expressed from the A allele
9 (which are smaller in size to distinguish between the A
10 (MvnI) and T (XbaI) alleles) were not cleaved by Rz907
11 - no cleavage products were observed. From top to
12 bottom, RNA from the T allele, the A allele and the two
13 cleavage products from the T allele are highlighted by
14 arrows.

1 Sequences

2

3 Sequence 1

4 The human rhodopsin cDNA in pCDNA3.

5

6 Sequence 2

7 The human rhodopsin cDNA in pCDNA3 with a base change
8 at a silent site (477).

9

10 Sequence 3

11 Mutant (Pro23Leu) human rhodopsin cDNA in pCDNA3.

12

13 Sequence 4

14 Rz10 cloned into pCDNA3. Note there is a one base
15 mismatch in one antisense arm of Rz10.

16

17 Sequence 5

18 Rz20 cloned into pCDNA3

19

20 Sequence 6

21 The mouse rhodopsin cDNA in pCDNA3.

22

23 Sequence 7

24 The mouse rhodopsin cDNA in pCDNA3 with a base change
25 at a silent site (1460).

26

27 Sequence 8

28 Rz33 cloned into pCDNA3

29

30 Sequence 9

31 The human peripherin cDNA in pCDNA3.

32

33 Sequence 10

34 The human peripherin DNA fragment with a base change at
35 a silent site (257).

36

(nuc. 271 of SEQ ID NO: 2)

(nuc. 216-218 of SEQ ID NO: 3)

(nuc. 190 of SEQ ID NO: 7)

(nuc. 332 of SEQ ID NO: 10)

1 Sequence 11

2 The human peripherin DNA fragment with a base change at
3 a silent site (359).

4 *(nuc. 468 of SEQ ID NO. 13)*

5 Sequence 12

6 Rz30 cloned into pCDNA3

7

8 Sequence 13

9 Rz31 cloned into pCDNA3

10

11 Sequence 14

12 Collagen 1A2 (A) sequence containing the A polymorphism
13 at position 907. (Note there is an additional
14 polymorphic site at position 902).

15

16 Sequence 15

17 Collagen 1A2 (B) sequence containing the T polymorphism
18 at position 907. (Note there is an additional
19 polymorphic site at position 902).

20

21 Sequence 16

22 Rz907 cloned into pCDNA3

23

24 Note:

25

26 The sequence quality was not good in the region of the
27 human peripherin 359 silent change - the sequencing
28 primer was too far from the target site to achieve good
29 quality sequence (Sequence 11).

(nucleotide 468 of SEQ ID NO. 13)

1 RESULTS

2
3 Human and mouse rhodopsin, human peripherin and human
4 collagen 1A2 cDNA clones were expressed *in vitro*.
5 Ribozymes targeting specific sites in the human and
6 mouse rhodopsin, human peipherin and human collagen 1A2
7 cDNAs were also expressed *in vitro*. cDNA clones were
8 cut with various restriction enzymes resulting in the
9 production of differently sized transcripts after
10 expression. This aided in differentiating between RNAs
11 expressed from unadapted and adapted cDNAs.
12 Restriction enzymes used to cut each clone, sizes of
13 resulting transcripts and predicted sizes of products
14 after cleavage by target ribozymes are given below in
15 Table 1. Exact sizes of expression products may vary
16 by a few bases from that estimated as there may be some
17 ambiguity concerning *inter alia* the specific base at
18 which transcription starts.

19
20 Example 121
22 A: Human Rhodopsin

23
24 The unadapted human rhodopsin cDNA and the human (SEQ ID NO: 1)
25 rhodopsin cDNA with a single nucleotide substitution in (SEQ ID NO: 2)
26 the coding sequence were cut with BstEII and expressed
27 *in vitro*. The single base change occurs at the third
28 base position or wobble position of the codon (at
29 position 477) and therefore does not alter the amino
30 acid coded by this triplet. The Rz10 clone was cut
31 with XbaI and expressed *in vitro*. Resulting ribozyme
32 and human rhodopsin RNAs were mixed with varying
33 concentrations of MgCl₂ to optimise cleavage of template
34 RNA by Rz10. A profile of human rhodopsin RNA cleavage
35 by Rz10 over time is given in Figure 1A. The
36 MgCl₂ curve profile used to test if adapted human

(MAC.271 of SEQ ID NO: 2)

1 rhodopsin transcripts could be cleaved by Rz10 is given
2 in Figure 2B. Unadapted and adapted human rhodopsin
3 cDNAs were cut with FspI and BstEII respectively,
4 expressed and mixed together with Rz10 RNA to test for
5 cleavage (Figure 1B) over time. Likewise, unadapted
6 and adapted human rhodopsin cDNAs were cut with AcyI
7 and BstEII respectively, both were expressed in vitro
8 and resulting transcripts mixed with Rz10 RNA at
9 varying MgCl₂ concentrations to test for cleavage
10 (Figure 2A, 2C). In all cases expressed RNAs were the
11 predicted size. Similarly in all cases unadapted
12 transcripts were cleaved into products of the predicted
13 size. Cleavage of unadapted human rhodopsin RNA was
14 almost complete - little residual uncleaved RNA
15 remained. In all cases adapted human rhodopsin RNAs
16 with a single base change at a silent site remained
17 intact, that is, ^{they were} ~~it was~~ not cleaved by Rz10. Clearly,
18 transcripts from the unadapted human rhodopsin cDNA are
19 cleaved by Rz10 while transcripts from the adapted
20 replacement gene which has been modified in a manner
21 which exploits the degeneracy of the genetic code are
22 protected from cleavage. It is worth noting that AcyI
23 enzyme cuts after the stop codon and therefore the
24 resulting RNA includes the complete coding sequence of
25 the gene.

26

27 B: Human Rhodopsin

28

29 Rz20 was cut with XbaI and expressed in vitro.
30 Similarly the rhodopsin cDNA containing a Pro23Leu
31 mutation was cut with BstEII and expressed in vitro.
32 Resulting RNAs were mixed and incubated at 37°C with
33 10mM ~~MgCl₂~~ ^{MgCl₂} for varying times. Rz20 was designed to
34 elicit mutation specific cleavage of transcripts
35 containing a Pro23Leu rhodopsin mutation. All
36 expressed products and cleavage products were the

1 correct size. Figure 3 demonstrates mutation specific
2 cleavage of the mutant RNA over time incubated at 37°C
3 with 10mM MgCl₂. Cleavage of mutant rhodopsin
4 transcripts by Rz10 which targets a ribozyme cleavage
5 site 3' of the site of the Pro23Leu mutation in
6 rhodopsin coding sequence was explored. The mutant
7 rhodopsin cDNA and Rz10 clones were cut with BstEII and
8 XbaI respectively and expressed *in vitro*. Resulting
9 RNAs were mixed and incubated with 10mM MgCl₂ for
10 varying times (Figure 4). All expressed products and
11 cleavage products were the correct size. Rz10 cleaved
12 mutant rhodopsin transcripts. Using a replacement gene
13 with a sequence change around the Rz10 cleavage site
14 which is at a wobble position we demonstrated in
15 Example 1A that transcripts from the replacement gene
16 remain intact due to absence of the Rz10 target site
17 (Figures 1B, 2A and 2B). Hence Rz10 could be used to
18 cleave mutant transcripts in a manner independent of
19 the disease mutation itself (that is, using this site)
20 while transcripts from the replacement gene which code
21 for the correct protein would remain intact and
22 therefore could supply the wild type protein.

23

24 Example 2

25 Mouse Rhodopsin

26 Rz33 was cut with XbaI and expressed *in vitro*.
27 Similarly the mouse rhodopsin cDNA was cut with
28 Eco47III and expressed *in vitro*. Resulting RNAs were
29 mixed and incubated with varying concentrations of
30 MgCl₂. All expressed products and cleavage products
31 were the correct size. Figure 5A demonstrates specific
32 cleavage of the mouse rhodopsin RNA over various MgCl₂
33 concentrations incubated at 37°C for 3 hours. Using a
34 replacement gene with a sequence change around the Rz33
35 cleavage site (TTT-->TCT) which is at a wobble position
36 we demonstrated that transcripts from the replacement

(inc. 189-191 of SEQ ID NO. 7)

1 gene remain intact due to absence of the Rz33 target
2 site (Figures 5B). Hence Rz33 could be used to cleave
3 mutant transcripts in a manner independent of the
4 disease mutation itself (that is, using this site)
5 while transcripts from the replacement gene which code
6 for the correct protein would remain intact and
7 therefore could supply the wild type protein.

8
9 Example 3

10 Human Peripherin

11 The unadapted human peripherin cDNA and two human
12 peripherin DNA fragments generated by PCR mutagenesis
13 with a single nucleotide substitution in the coding
14 sequence were cut with BglII and AvrII respectively and
15 expressed in vitro. The single base changes in the
16 adapted DNAs occur at third base positions or wobble
17 positions of the codon (at position 257, and 359) and
18 therefore do not alter the amino acid coded by these
19 triplets. The Rz30 and Rz31 clones were cut with XbaI
20 and expressed in vitro. Resulting ribozymes and
21 unadapted human rhodopsin RNAs were mixed with varying
22 concentrations of MgCl₂ to optimise cleavage of template
23 RNA by Rz30 and Rz31. Profiles of human peripherin RNA
24 cleavage by Rz30 over various MgCl₂ concentrations and
25 over time are given in Figure 6. Similarly profiles of
26 human peripherin RNA cleavage by Rz31 over various MgCl₂
27 concentrations and over time are given in Figure 7. In
28 all cases expressed RNAs were the predicted size.
29 Similarly in all cases unadapted transcripts were
30 cleaved into products of the predicted size. Adapted
31 human rhodopsin RNAs were mixed together with Rz30 and
32 Rz31 RNA over various MgCl₂ concentrations to test if
33 adapted human peripherin transcripts could be cleaved
34 by Rz30 and Rz31 (Figures 6 + 7). Expressed RNAs were
35 the predicted size. In all cases adapted human
36 peripherin RNAs with single base changes at silent

(NMC 468 of
SEQ ID NO 13)

(NMC 332 of
SEQ ID NO 10)

1 sites remained intact, that is, they were not cleaved
2 by Rz30 or Rz31. Clearly, transcripts from the
3 unadapted human peripherin cDNA are cleaved by Rz30 and
4 Rz31 while transcripts from the adapted replacement
5 DNAs which have been modified in a manner which
6 exploits the degeneracy of the genetic code are
7 protected from cleavage.

8

9 Example 4

10 Human Collagen 1A2

11 Rz907 clones targeting a polymorphic site in human
12 collagen 1A2 sequence was cut with XbaI and expressed
13 in vitro. The human collagen 1A2 cDNA clones (A and B)
14 containing two allelic forms of a polymorphism in the
15 coding sequence of the gene at positions 907 were cut
16 with MvnI and XbaI respectively, expressed in vitro and
17 RNAs mixed together with Rz907 RNA to test for cleavage
18 of transcripts by this ribozyme. All expressed
19 transcripts were of the predicted sizes. RNAs were
20 mixed with varying concentrations of MgCl₂ to optimise
21 cleavage of RNAs by Rz907 (Figure 8). Notably the
22 majority of the RNA transcripts from human collagen 1A2
23 (B) which has a T nucleotide at position 907 is cleaved
24 by Rz907 (Figure 8). This allelic form of the gene has
25 a ribozyme cleavage site at 906-908. Notably the
26 situation is reversed with transcripts from human
27 collagen 1A2 (A) where in this allelic form of the gene
28 due to the nature of the polymorphism present at
29 position 907 the ribozyme cleavage site has been lost.
30 In contrast to transcripts from human collagen (B),
31 transcripts from human collagen (A) were protected from
32 cleavage by Rz907 due to the alteration in the sequence
33 around the ribozyme cleavage site (Figure 8). Cleavage
34 of collagen 1A2 (B) by Rz907 was efficient which is
35 consistent with 2-D predictions of RNA open loop
36 structures for the polymorphism - in the allele

→ nuc.
17 6 87
SFB 10 NO 17

1 containing the Rz907 ribozyme cleavage site, the target
2 site is found quite consistently in an open loop
3 structure. This polymorphism found in an open loop
4 structure of the transcript clearly demonstrates the
5 feasibility and utility of using the degeneracy of the
6 genetic code in the suppression of an endogenous gene
7 (either suppressing both alleles or a single allele at
8 a polymorphic site) and restoration of gene expression
9 using a gene which codes for the same protein but has
10 sequence modifications at third base wobble positions
11 which protect the replacement gene from suppression.

TABLE 1

	Restriction Enzyme	RNA Size	Cleavage Products
Example 1			
Human rhodopsin	BstEII	851 bases	287+564 bases
	AcyI	1183 bases	287+896 bases
	FspI	309 bases	287+22
Adapted Human rhodopsin	BstEII	851 bases	
Human rhodopsin			
Pro-Leu	BstEII	851 bases	170+681 (Rz20)
Human rhodopsin			
Pro-Leu	BstEII	851 bases	287+564 (Rz10)
Rz10	XbaI	52 bases	
Rz20	XbaI	52 bases	
(Table 1; Sequences 1-5 ; Figures 1-4)			
SEB ID NOS:			
Example 2			
Mouse rhodopsin	Eco47III	774 bases	400+374
Adapted mouse rhodopsin	Eco47III	774 bases	
Rz33	XbaI	52 bases	
(Table 1; Sequences 6-9; Figure 5)			
SEB ID NOS:			
Example 3			
Human peripherin	BglII	545 bases	315+230 (Rz30)
Human peripherin	BglII	545 bases	417+128 (Rz31)
Adapted human peripherin	AvrII	414 bases	
Adapted human peripherin	BglII	545 bases	
Rz30	XbaI	52 bases	
Rz31	XbaI	52 bases	
(Table 1; Sequences 10-14; Figures 6+7)			
SEB ID NOS: 10, 13-15)			
Example 4			
Human Collagen 1A2			
(B) -Rz907	XbaI	888 bases	690+198 bases
Human Collagen			
1A2 (A)	MvnI	837 bases	
Rz907	XbaI	52 bases	
(Table 1; Sequences 14-16; Figure 8)			
SEB ID NOS: 16-18			

TABLE 2

A: Rhodopsin mutations tested to assess if the predicted open loop RNA structure containing the Rz10 target site (475-477) remains intact in mutant transcripts.

Rhodopsin mutation	RNA open loop targeted by Rz10
Pro 23 Leu	Intact
Gly 51 Val	Intact
Thr 94 Ile	Intact
Gly 188 Arg	Intact
Met 207 Arg	Intact
Ile del 255	Intact

B: Utilisation of the degeneracy of the genetic code.
Ribozyme cleavage sites are underlined

Human rhodopsin

Unadapted sequence	475-477 TAC <u>GTC</u> ACC GTC CAG (SEQ ID NO. 19) Val
Adapted sequence	475-477 TAC GTG ACC GTC CAG (SEQ ID NO. 20) Val

Mouse rhodopsin

Unadapted sequence	1459-1461 AAT <u>TTT</u> TAT GTG CCC (SEQ ID NO. 21) Phe
Adapted sequence	1459-1461 AAT TTC TAT GTG CCC (SEQ ID NO. 22) Phe

Human peripherin

Unadapted sequence	255-257 GCG <u>CTA</u> CTG AAA GTC (SEQ ID NO. 23) Leu
Adapted sequence	255-257 GCG CTG CTG AAA GTC (SEQ ID NO. 24) Leu
Unadapted sequence	357-359 AGC <u>CTA</u> GGA CTG TTC (SEQ ID NO. 25) Leu
Adapted sequence	357-359 AGC CTG GGA CTG TTC (SEQ ID NO. 26) Leu

Human type I collagen 1A2

Sequence (B)	906-908 GCT <u>GGT</u> CCC GCC GGT (SEQ ID NO. 27) Gly
--------------	--

Sequence (A) 906-908
GCT GGA CCC GCC GGT (≤ 60 ID NO. 28)
Gly

1 DISCUSSION

2

3 In the examples outlined above, RNA was expressed from
4 cDNAs coding for four different proteins: human and
5 mouse rhodopsin, human peripherin and human type I
6 collagen 1A2. Rhodopsin and peripherin have been used
7 to exemplify the invention for retinopathies such as
8 adRP - suppression effectors have been targeted to the
9 coding sequences of these genes. In the case of the
10 human collagen 1A2 gene a naturally occurring
11 polymorphism has been used to demonstrate the invention
12 and the potential use of the invention for disorders
13 such as OI - however non-polymorphic regions of the
14 collagen 1A2 gene could be used to achieve suppression.
15 The suppression effectors of choice in the invention
16 have been hammerhead ribozymes with antisense flanks to
17 define sequence specificity. Hammerhead ribozymes
18 require NUX cleavage sites in open loop structures of
19 RNA. Notably, other suppression effectors could be
20 utilised in the invention and may lead to a more
21 flexible choice of target sequences for suppression.
22 Transcripts expressed from all four genes have been
23 significantly attacked in vitro using suppression
24 effectors directed towards target cleavage sites. In
25 all four examples the ribozymes directed to cleavage
26 sites were successful in cleaving target RNAs in the
27 predicted manner. Antisense complementary to sequences
28 surrounding the cleavage sites was used successfully
29 to elicit binding and cleavage of target RNAs in a
30 sequence specific manner. Additionally, transcripts
31 from replacement genes, modified using the degeneracy
32 of the genetic code so that they code for wild type
33 protein, were protected fully from cleavage by

1 ribozymes.

2

3 The utility of an individual ribozyme designed to
4 target an NUX site in an open loop structure of
5 transcripts from a gene will depend in part on the
6 robust nature of the RNA open loop structure when
7 various deleterious mutations are also present in the
8 transcript. To evaluate this, we analysed RNAPlotFold
9 data for six different adRP causing mutations in the
10 rhodopsin gene. For each of these, the large RNA open
11 loop structure which is targeted by Rz10 was predicted
12 to be maintained in the mutant transcripts (Table 2A).
13 This is clearly demonstrated in example 1B (Figure 3)
14 using a Pro23Leu rhodopsin mutation. Rz10 clearly
15 cleaves the mutant transcript effectively *in vitro*.
16 The Pro23Leu mutation creates a ribozyme cleavage site
17 and can be cleaved *in vitro* by Rz20 a ribozyme
18 specifically targeting this site - however this is not
19 the case for many mutations. In contrast we have shown
20 that the Rz10 ribozyme cleavage site is available for
21 different mutant rhodopsins and could potentially be
22 used to suppress multiple mutations using a suppression
23 and replacement approach.

24

25 In some cases lowering RNA levels may lead to a
26 parallel lowering of protein levels however this may
27 not always be the case. In some situations mechanisms
28 may prevent a significant decrease in protein levels
29 despite a substantial decrease in levels of RNA.
30 However in many instances suppression at the RNA level
31 has been shown to be effective (see prior art). In some
32 cases it is thought that ribozymes elicit suppression
33 not only by cleavage of RNA but also by an antisense
34 effect due to the antisense arms of the ribozyme
35 surrounding the catalytic core.

36

1 In all examples provided ribozymes were designed to
2 cleave at specific target sites. Target sites for four
3 of the ribozymes utilised were chosen in open loop
4 structures in the coding regions of transcripts from
5 three retinal genes (human and mouse rhodopsin and
6 human peripherin). In all cases sequence specific
7 cleavage was obtained at the target cleavage sites
8 (Figs 1-7). Target sites were chosen in open loop
9 structures to optimise cleavage. Additionally target
10 sites were chosen such that they could be obliterated
11 by single nucleotide changes at third base wobble
12 positions and therefore would code for the same amino
13 acid (Table 2B). In turn this enabled the generation
14 of replacement genes with single nucleotide alterations
15 which code for wild type protein. In all cases tested
16 transcripts from replacement genes were protected from
17 cleavage by ribozymes. Further modifications could be
18 made to replacement genes in wobble positions, for
19 example, to limit the binding ability of the antisense
20 arms flanking the ribozyme catalytic core. The examples
21 provided for rhodopsin and peripherin involve
22 suppression of expression of both disease and wild type
23 alleles of a retinal gene and restoration of the wild
24 type protein using a replacement gene. However, there
25 may be situations where single alleles can be targeted
26 specifically or partially specifically
27 (PCT/GB97/00574).

28
29 In one example, human collagen 1A2, Rz907 was used to
30 target a naturally occurring polymorphic site at amino
31 acid 187, (GGA (glycine) --> GGT (glycine), located in
32 an open loop structure from the predicted 2-D
33 conformations of the transcript (Figure 8, Table 2B).
34 The ribozyme Rz907 cleaved transcripts containing the
35 GGT sequence but transcripts with GGA were protected
36 from cleavage. Transcripts from both alleles of

1 individuals homozygous for the GGT polymorphism could
2 be cleaved by Rz907 whereas in the case of
3 heterozygotes cleavage could be directed to single
4 alleles (in particular to alleles containing
5 deleterious mutations PCT/GB97/00574). In both
6 situations replacement genes could have the sequence
7 GGA and therefore would be protected from cleavage by
8 Rz907. The presence of many such naturally occurring
9 silent polymorphisms highlights that replacement genes
10 could be modified in a similar fashion in wobble
11 positions and should produce in most cases functional
12 wild type protein. Multiple modifications could be
13 made to replacement genes at wobble positions
14 which would augment protection from suppression
15 effectors. For example, in situations where antisense
16 nucleic acids were used for suppression, transcripts
17 from replacement genes with multiple modifications at
18 third base positions would be protected partially or
19 completely from antisense binding.

20
21 In all four examples provided transcripts from cDNA
22 clones were cleaved in vitro in a sequence specific
23 manner at ribozyme cleavage sites. Additionally one
24 base of the ribozyme cleavage site occurs at a wobble
25 position and moreover can be altered so as to eliminate
26 the cleavage site. Ribozyme cleavage sites in the
27 examples given were destroyed by changing nucleotide
28 sequences so that the consensus sequence for ribozyme
29 cleavage sites was broken. However it may be
30 that in some cases the cleavage site could be destroyed
31 by altering the nucleotide sequence in a manner that
32 alters the 2-D structure of the RNA and destroys the
33 open loop structure targeted by the ribozyme. cDNAs or
34 DNA fragments with altered sequences in the regions
35 targeted by ribozymes were generated. RNAs expressed
36 from these cDNAs or DNA fragments were protected

1 entirely from cleavage due to the absence of the
2 ribozyme cleavage site for each of the ribozymes
3 tested. Of particular interest is the fact that a
4 single nucleotide alteration can obliterate a ribozyme
5 target site, thereby preventing RNA cleavage. Although
6 ribozymes have been used in the demonstration of the
7 invention, other suppression effectors could be used to
8 achieve gene silencing. Again replacement genes with
9 altered sequences (at third base wobble positions)
10 could be generated so that they are protected partially
11 or completely from gene silencing and provide the wild
12 type (or beneficial) gene product.

13
14 As highlighted before in the text, using the invention
15 the same method of suppression (targeting coding
16 sequences of a gene) and where necessary gene
17 replacement (using a replacement gene with a sequence
18 modified in third base positions to restore gene
19 expression) may be used as a therapeutic approach for
20 many different mutations within a given gene. Given
21 the continuing elucidation of the molecular
22 pathogenesis of dominant and polygenic diseases the
23 number of targets for this invention is rapidly
24 increasing.

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1 CLAIMS

- 2
- 3 1. A strategy for suppressing at least a part of the
- 4 coding region of an endogenous gene and replacing
- 5 the suppressed gene sequence with a nucleic acid
- 6 sequence which differs from the endogenous gene.
- 7
- 8 2. A strategy as claimed in Claim 1 wherein the
- 9 suppressing agent comprises at least one
- 10 suppressor from the group comprising antisense
- 11 nucleic acid, DNA capable of forming triple helix
- 12 or ribozymes to the endogenous gene.
- 13
- 14 3. A strategy as claimed in Claim 1 or 2 wherein the
- 15 replacement nucleic acid sequence encodes at least
- 16 part of a gene product and does not interact with
- 17 the suppressing agent.
- 18
- 19 4. A strategy as claimed in any of the preceding
- 20 claims wherein the replacement nucleic acid
- 21 sequence comprises amino acid codons which encode
- 22 at least part of the gene product, and differ from
- 23 the gene to be suppressed in at least the third
- 24 base of at least one of the codons.
- 25
- 26 5. A medicament comprising either one or both of a
- 27 gene suppressing agent and a nucleic acid encoding
- 28 at least part of a replacement gene product for
- 29 use in a strategy as claimed in any of the
- 30 preceding claims.
- 31
- 32 6. A medicament comprising a nucleic acid sequence
- 33 encoding at least part of a gene product wherein
- 34 the sequence differs from an endogenous gene in at
- 35 least the third base of at least one amino acid
- 36 codon.

- 1 7. A strategy for suppressing specifically or
2 partially specifically an endogenous gene and
3 introducing a replacement gene, said strategy
4 comprising the steps of:
5
6 1. providing suppressing nucleic acids or other
7 suppression effectors able to bind to an
8 endogenous gene, gene transcript or gene
9 product to be suppressed and
10
11 2. providing genomic DNA or cDNA (complete or
12 partial) encoding a replacement gene wherein
13 the suppressing nucleic acids are unable to
14 bind to equivalent regions in the genomic DNA
15 or cDNA to prevent expression of the
16 replacement gene.
17
18 8. A strategy as claimed in Claim 8 wherein the
19 coding sequence of replacement nucleic acids has
20 been altered to prevent or reduce efficiency of
21 suppression.
22
23 9. A strategy as claimed in Claim 8 or 9 wherein
24 replacement nucleic acids have modifications in
25 one or more third base (wobble) positions such
26 that replacement nucleic acids still code for the
27 wild type or equivalent amino acids.

Diagram 1

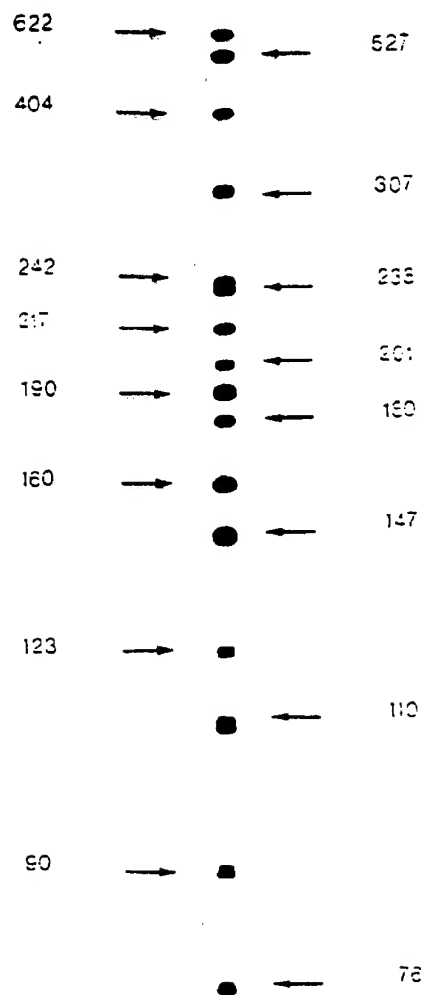


Figure 1 A + B

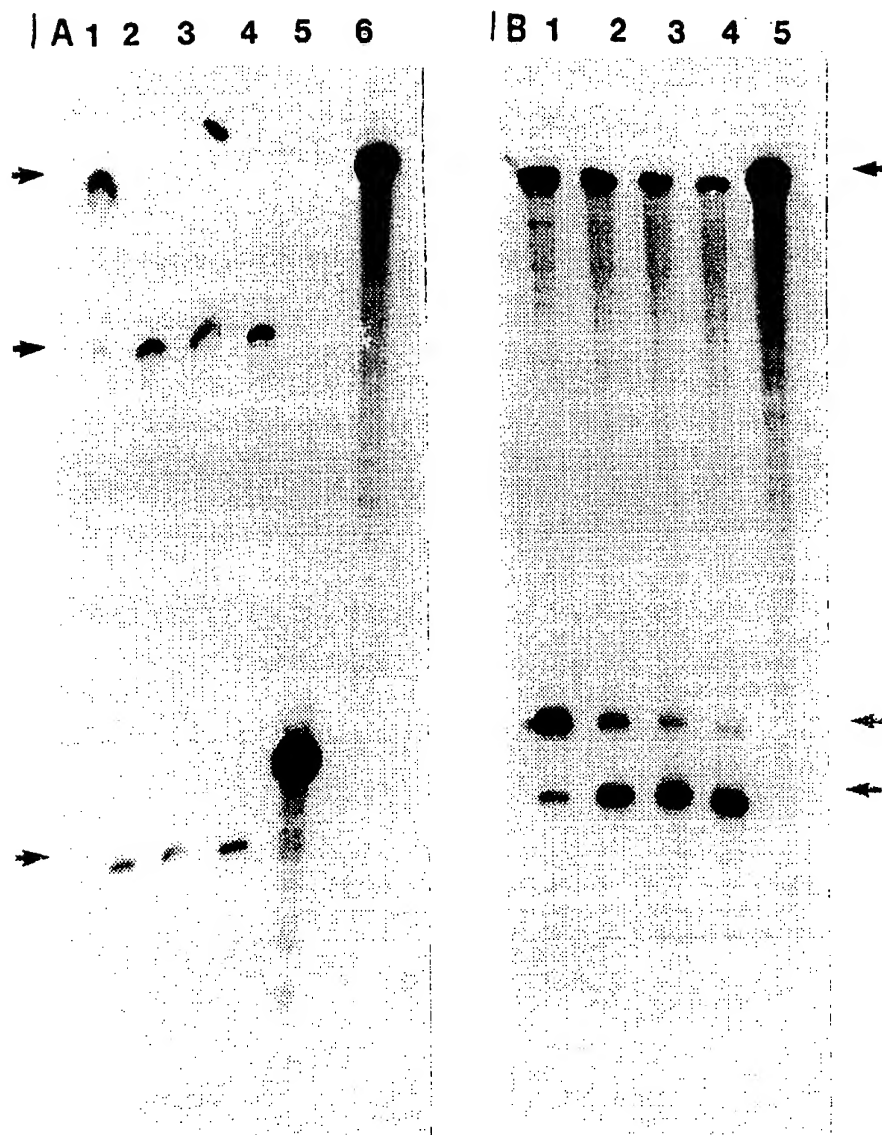


Figure 2 A+B

2 A 1 2 3 4 5 6 2B 1 2 3 4 5

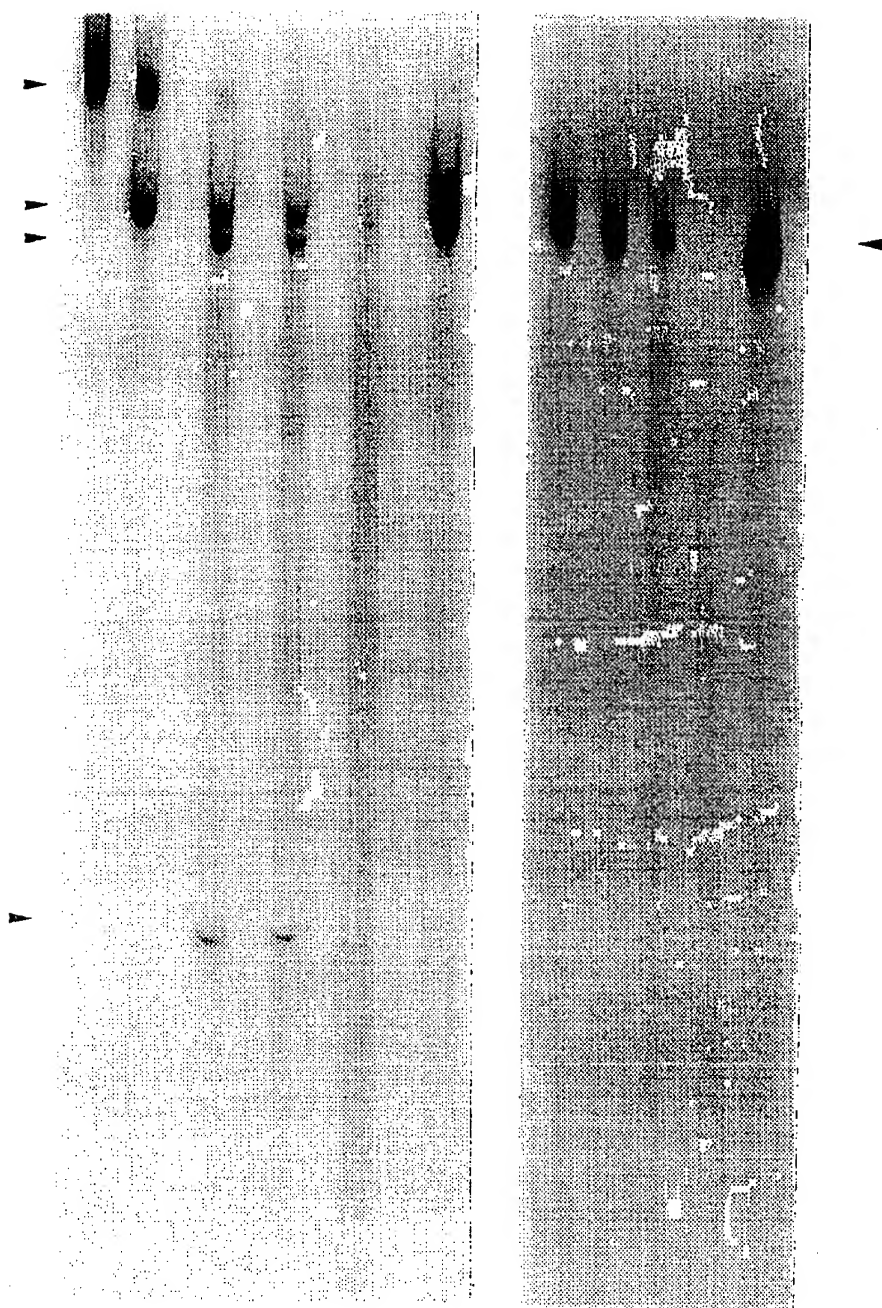


Figure 2c

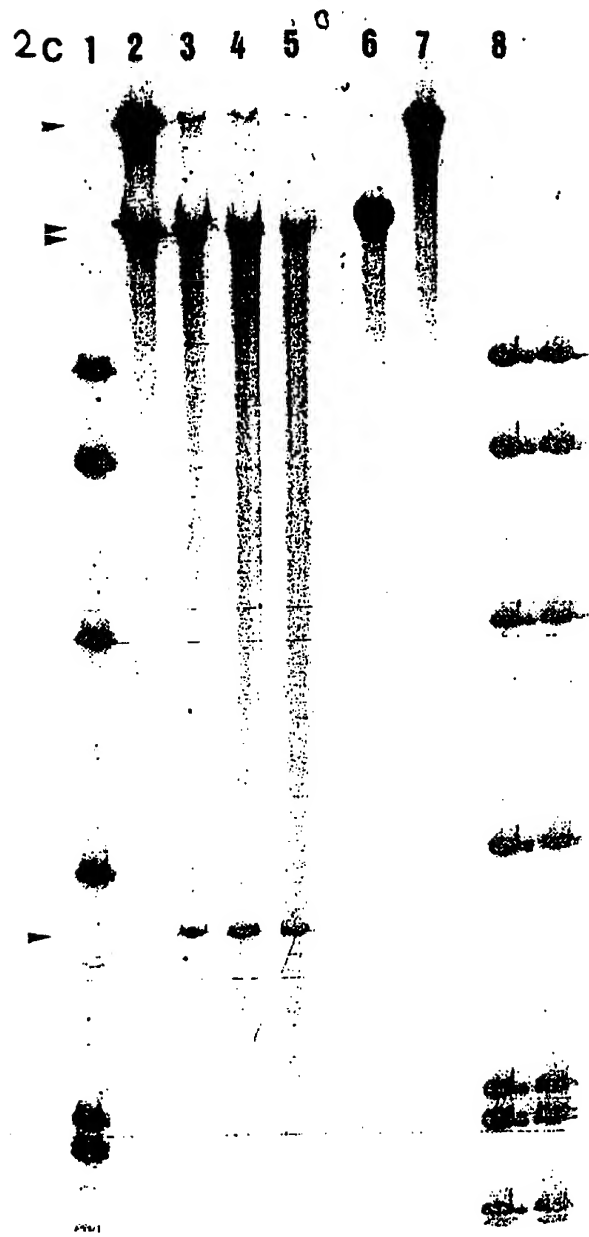


Figure 3

1 2 3 4 5 6 7 8

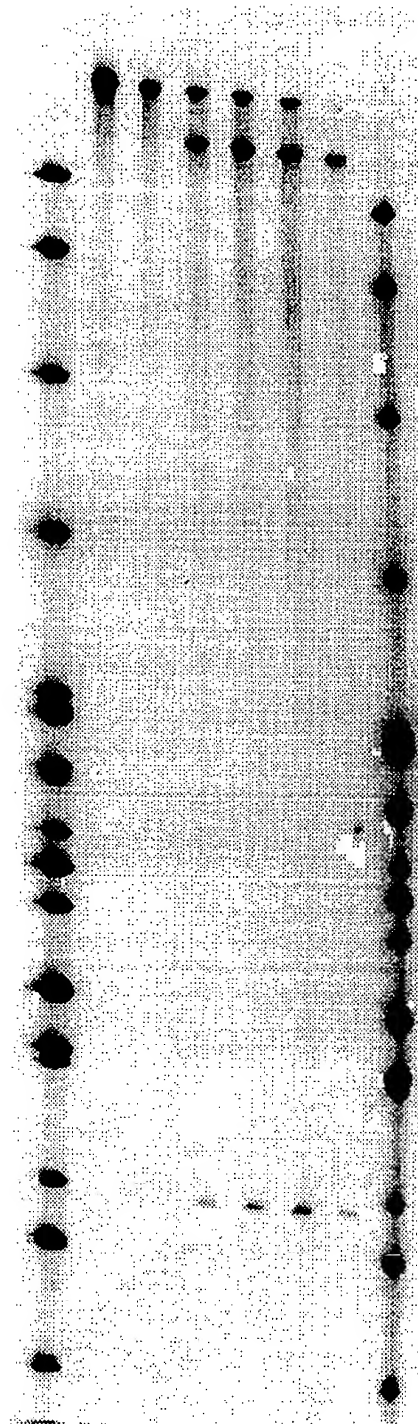


Figure 4

1 2 3 4 5 6 7 8

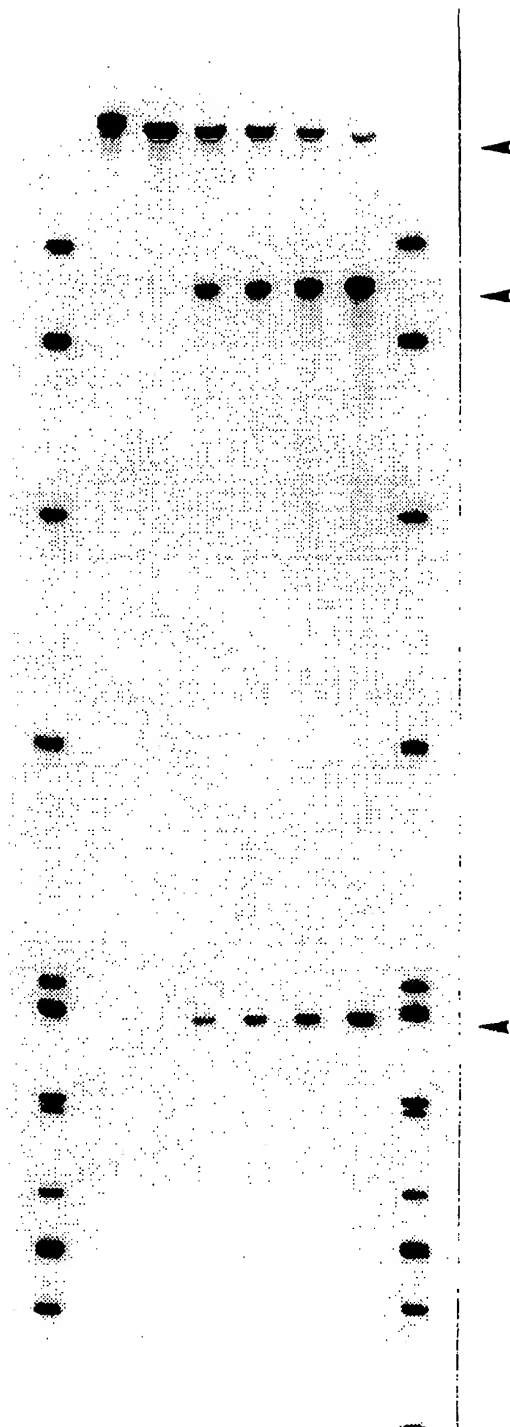


Figure 5 A

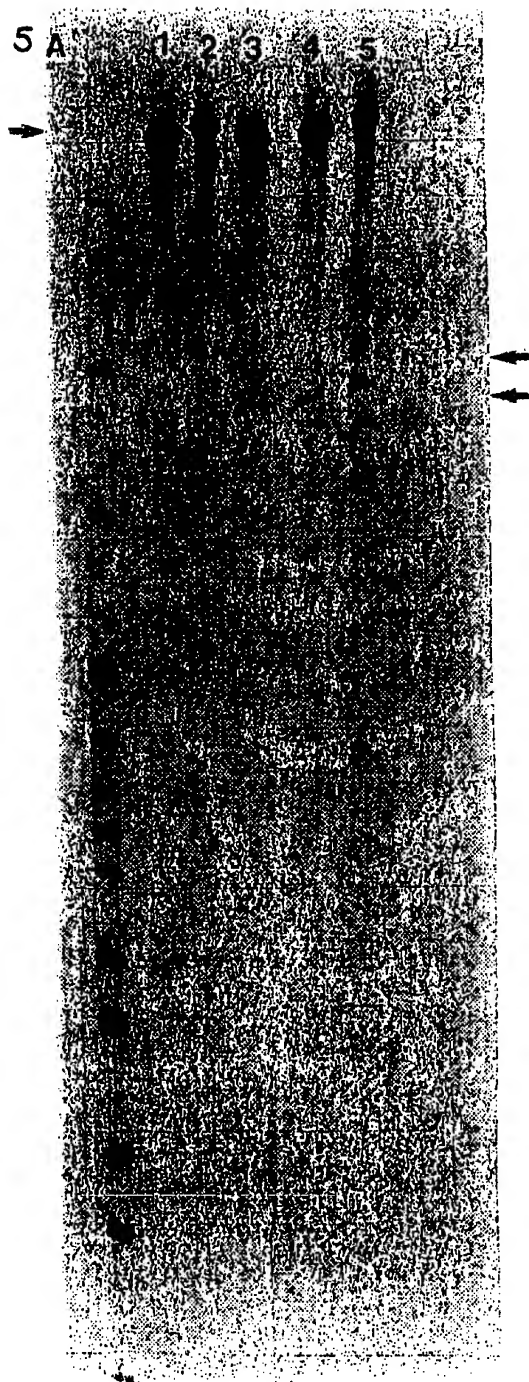


Figure 5B

5 B 1 2 3 4 5 6

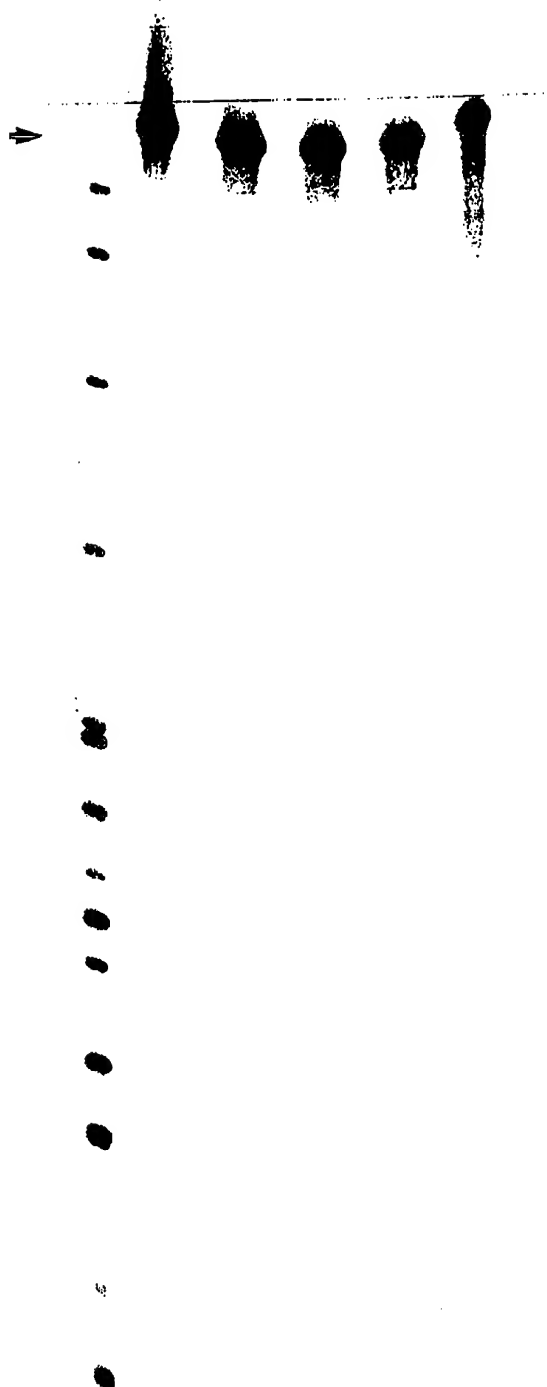


Figure 6 A+B

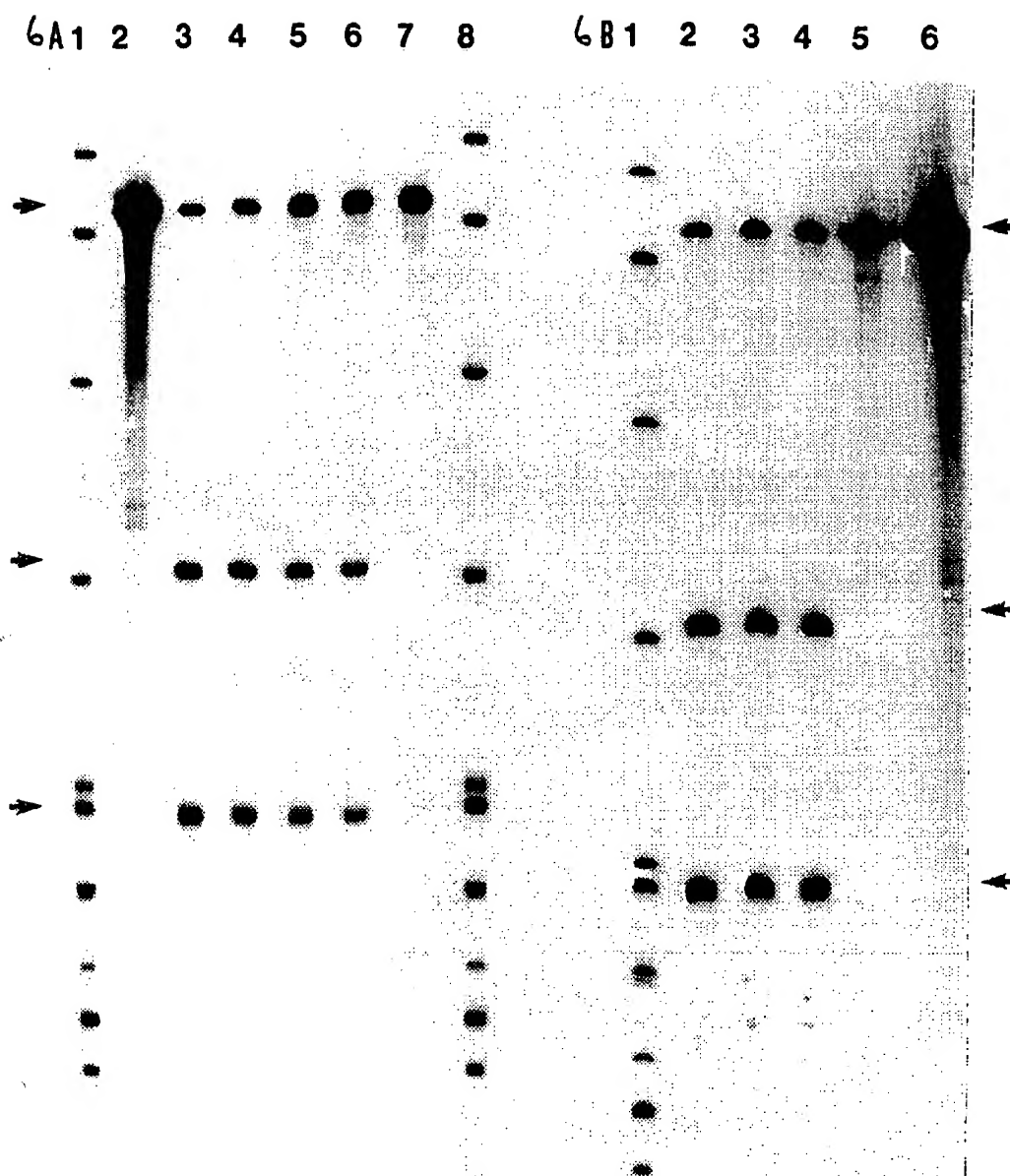


Figure 6C

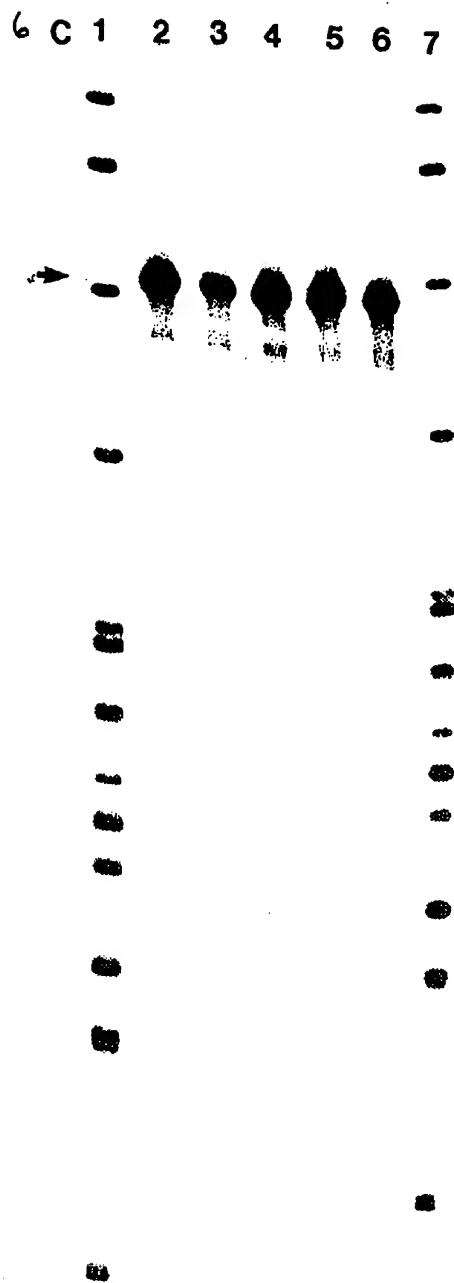


Figure 6D

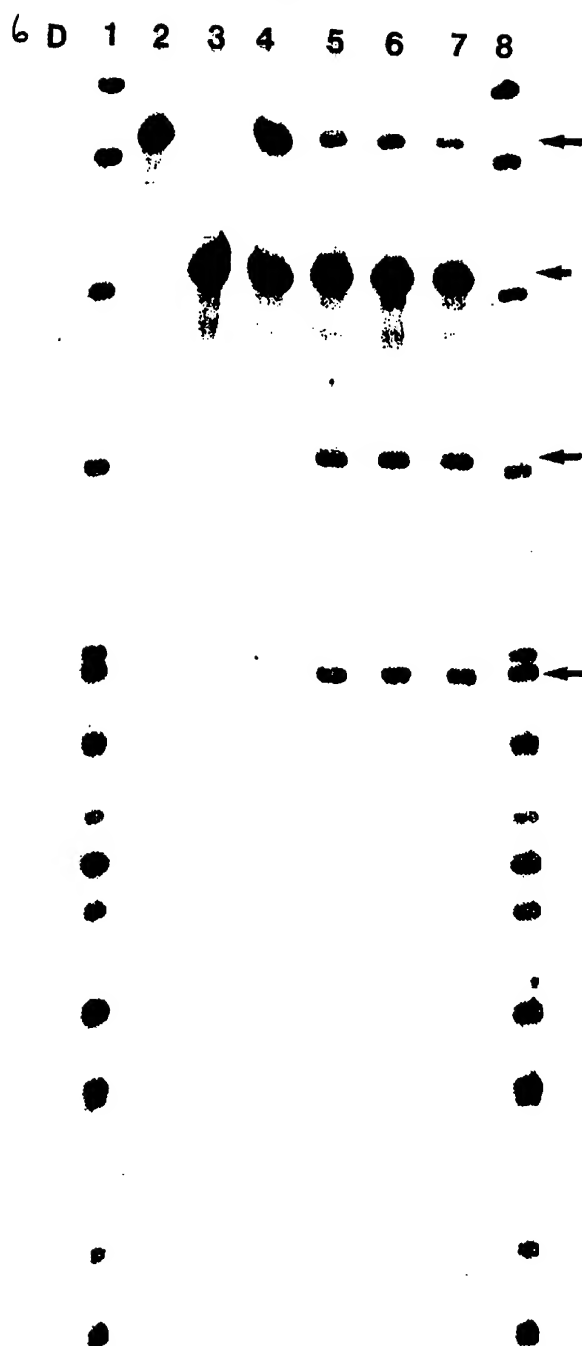
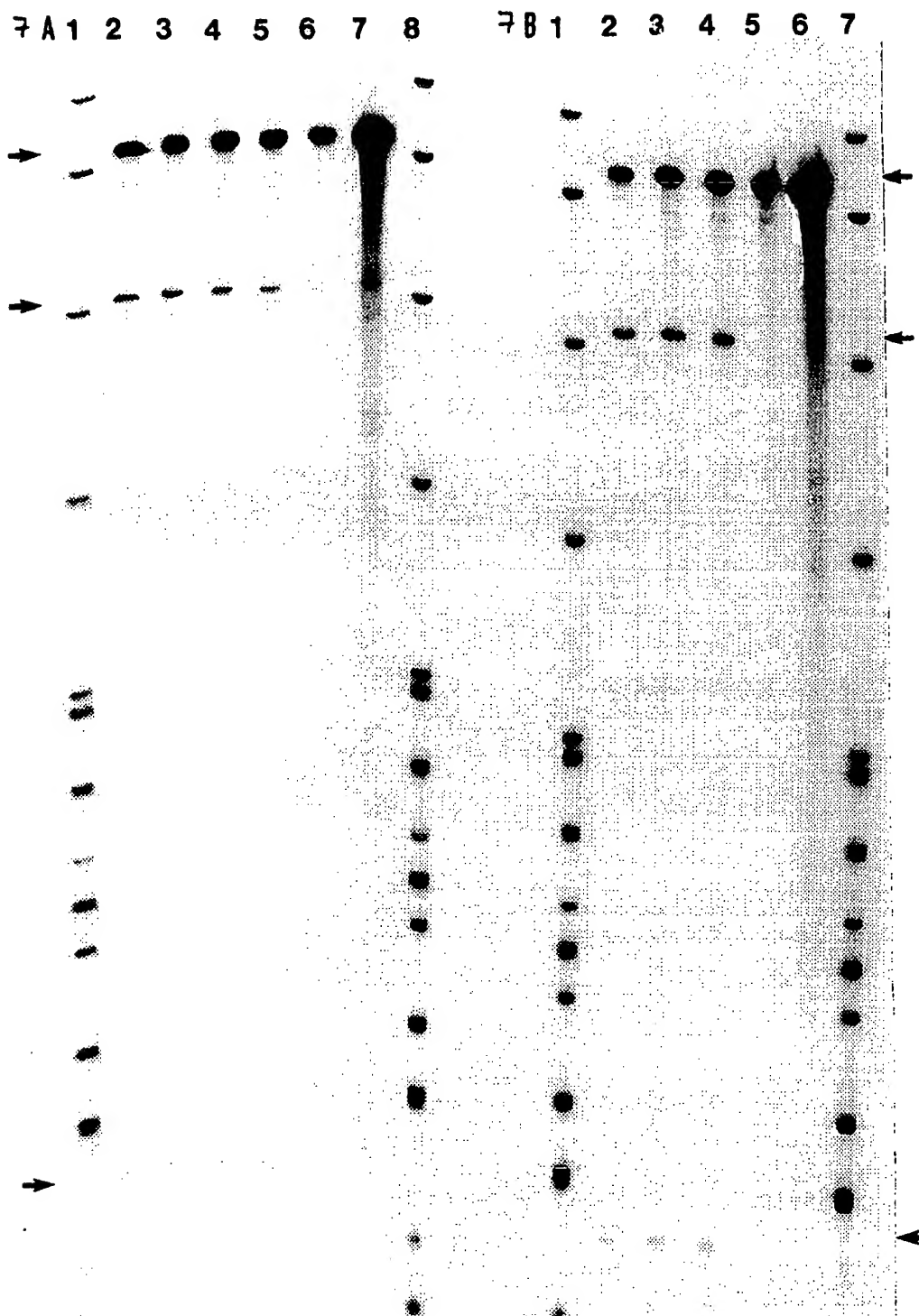


Figure 7 A + B



12/30

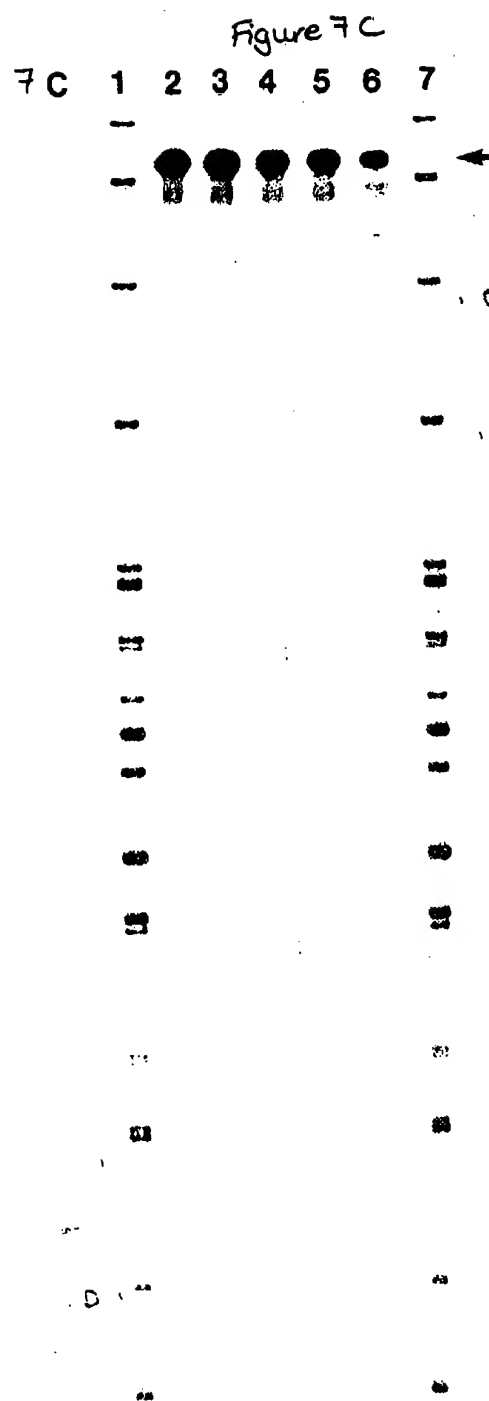
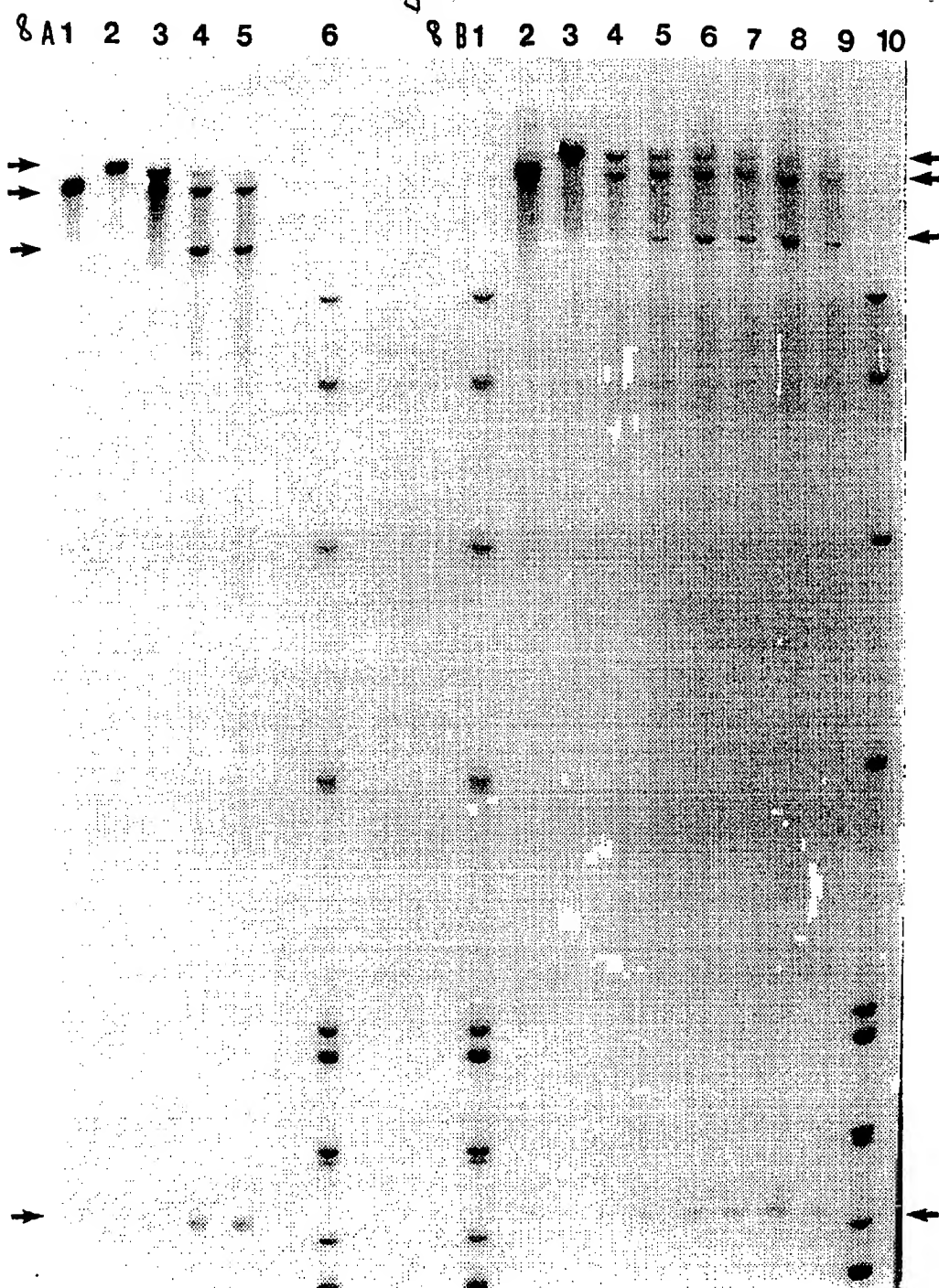
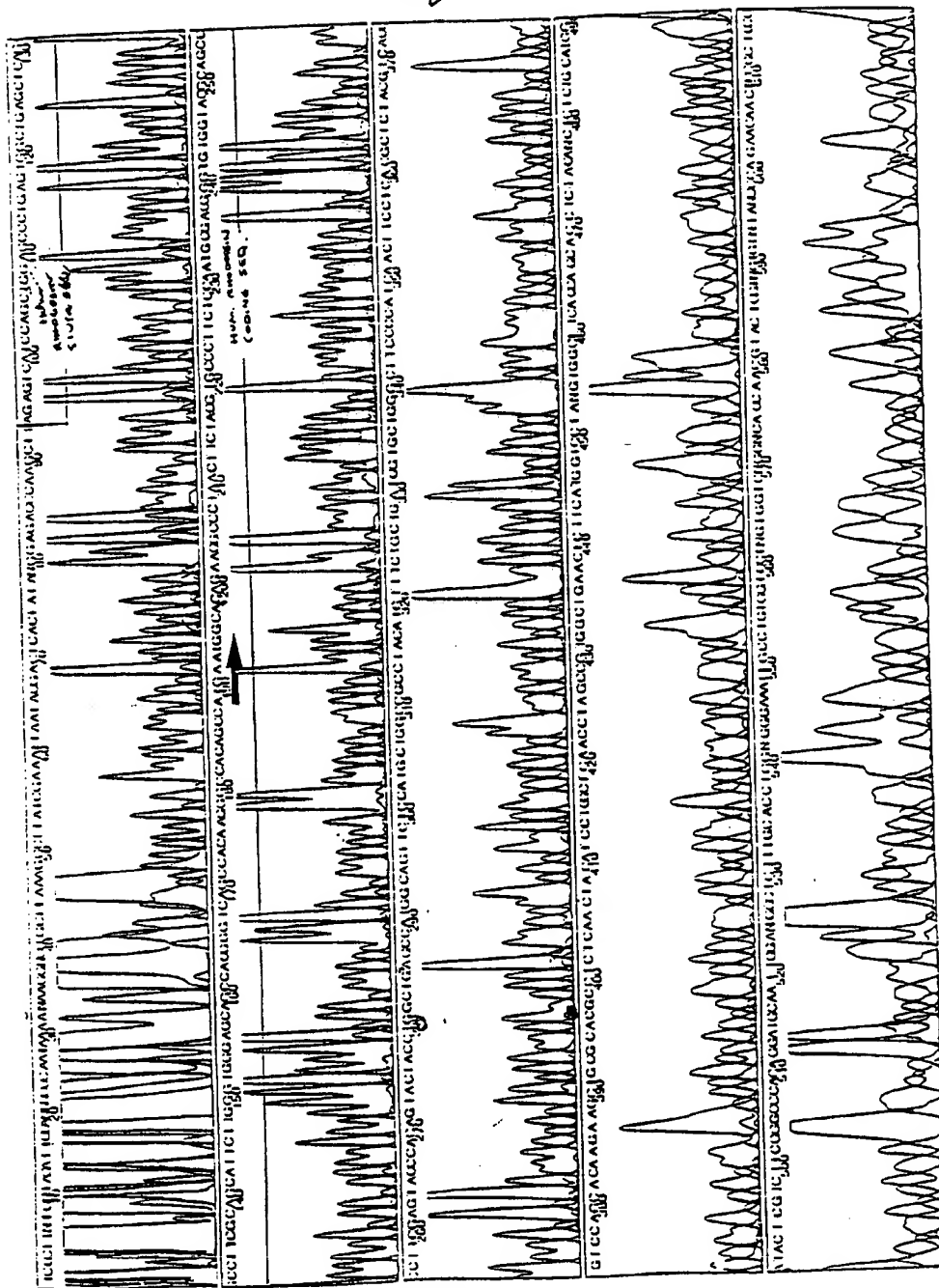
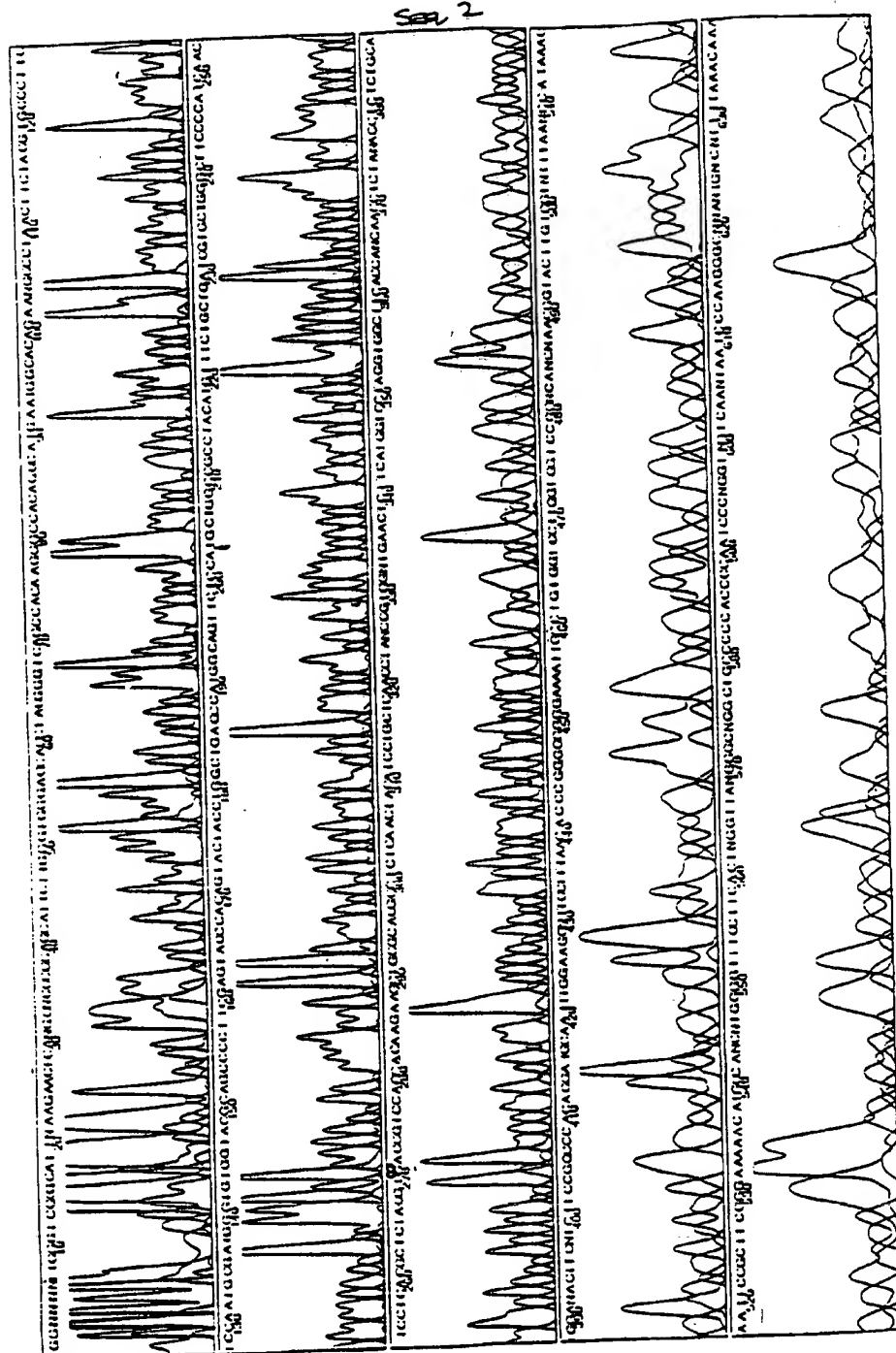


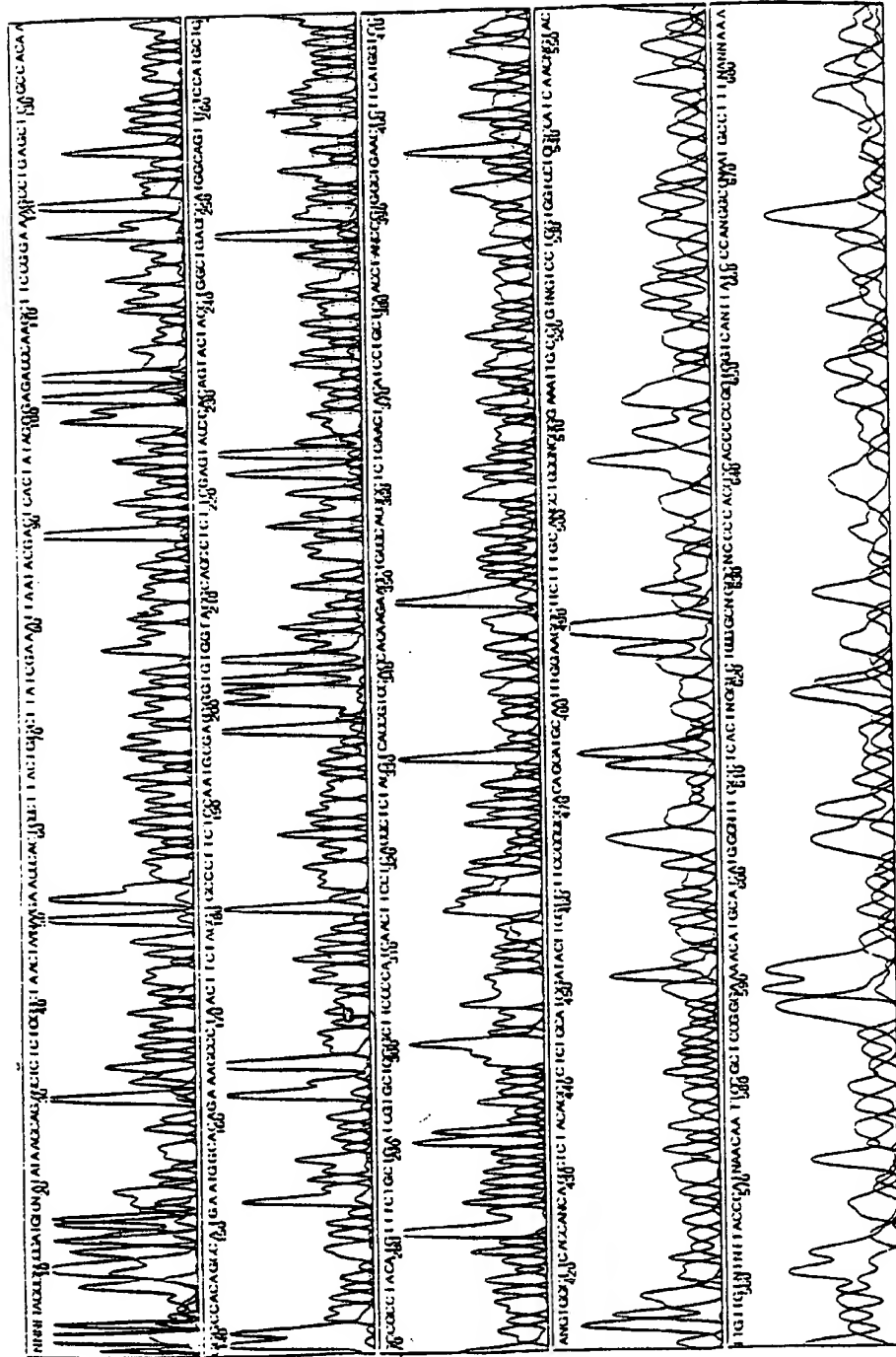
Figure 8 A + B



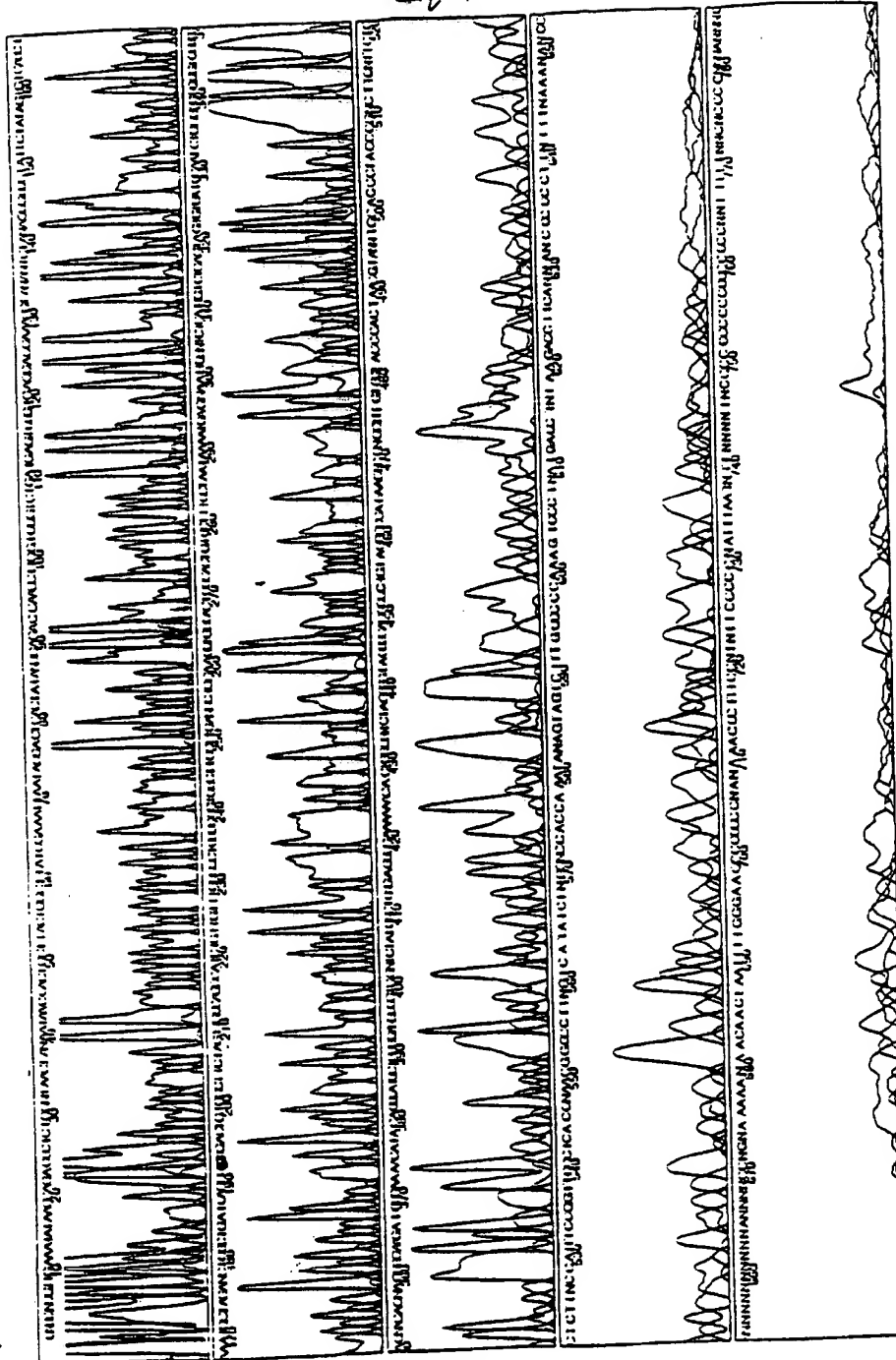




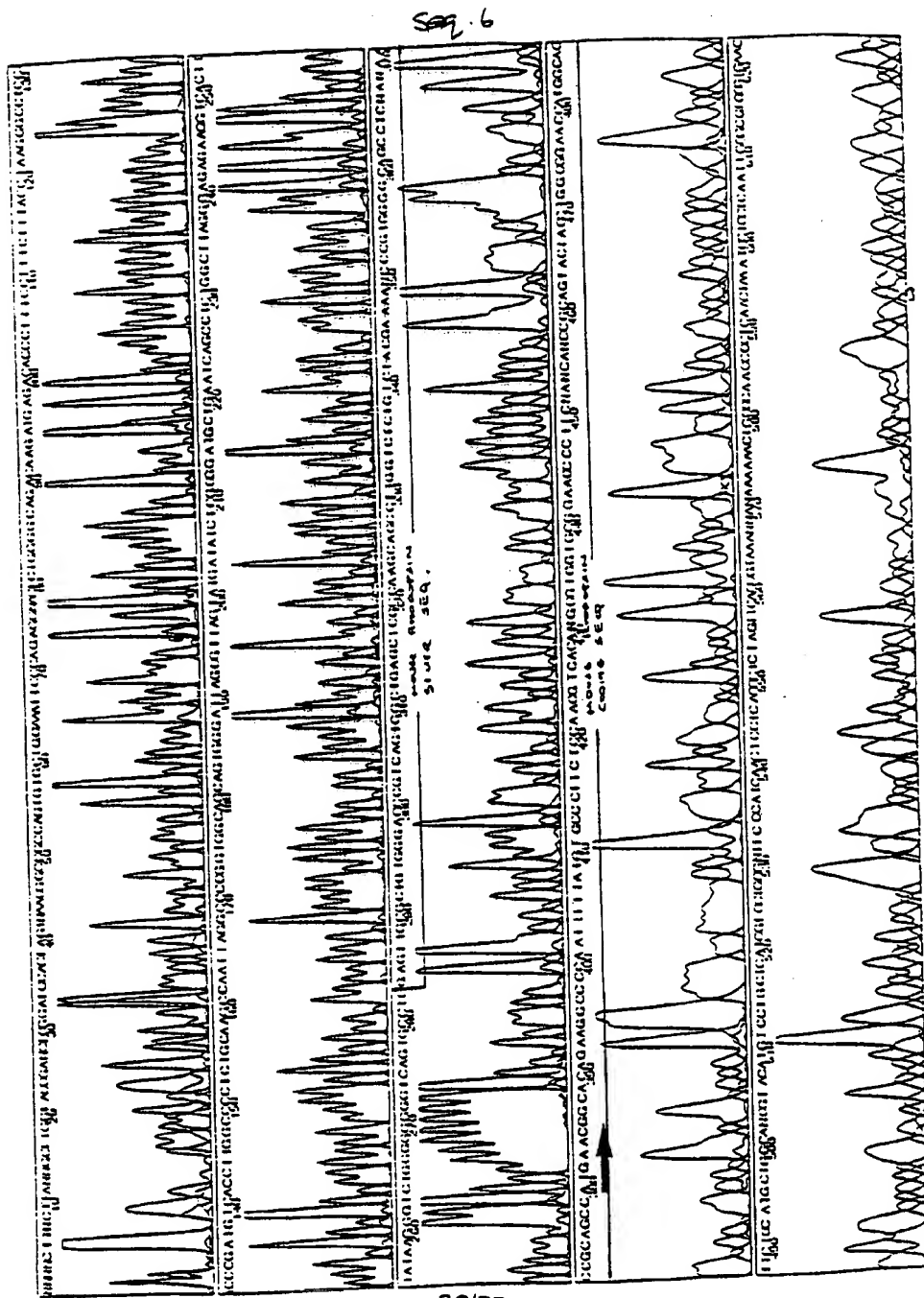
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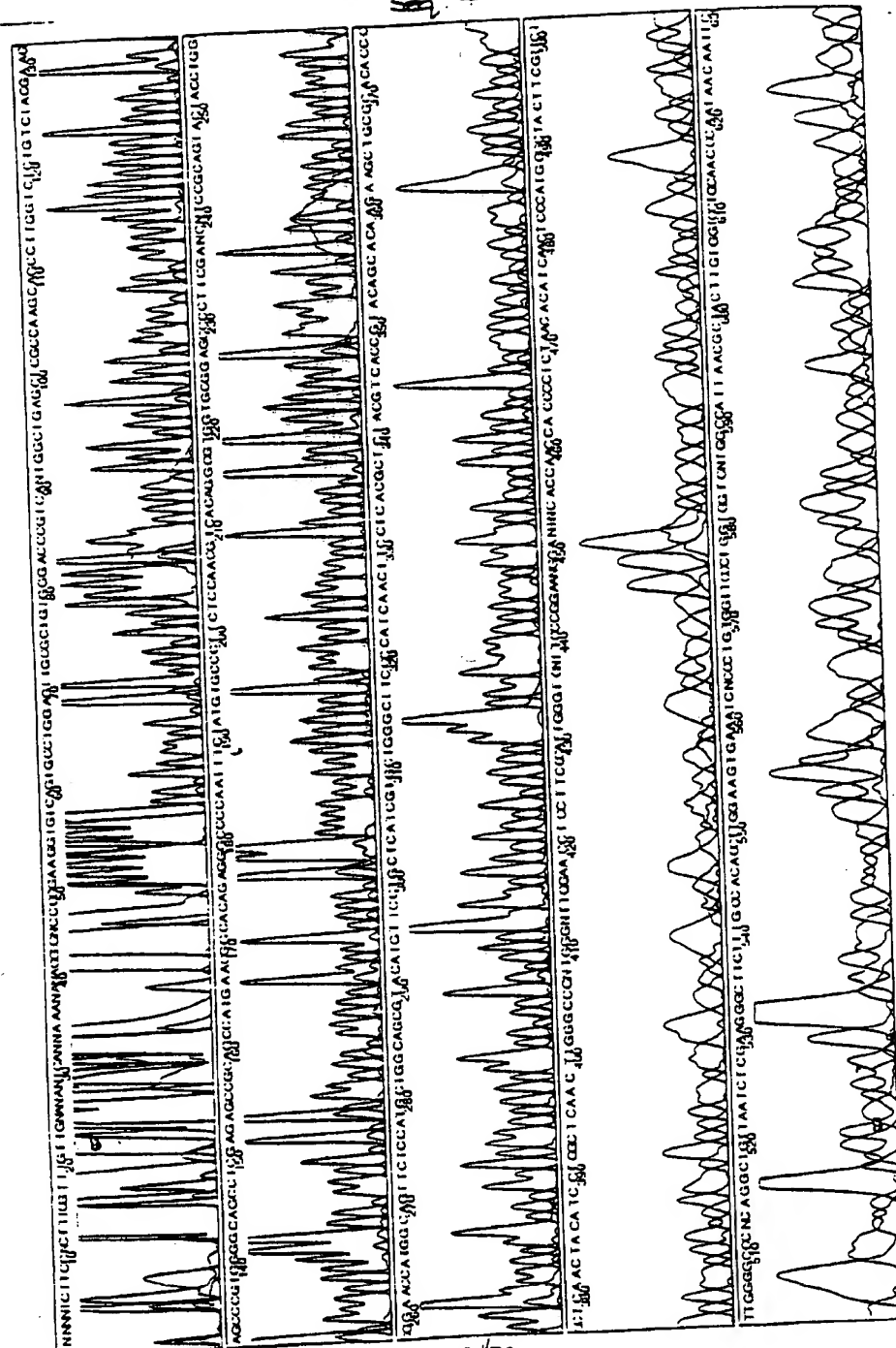


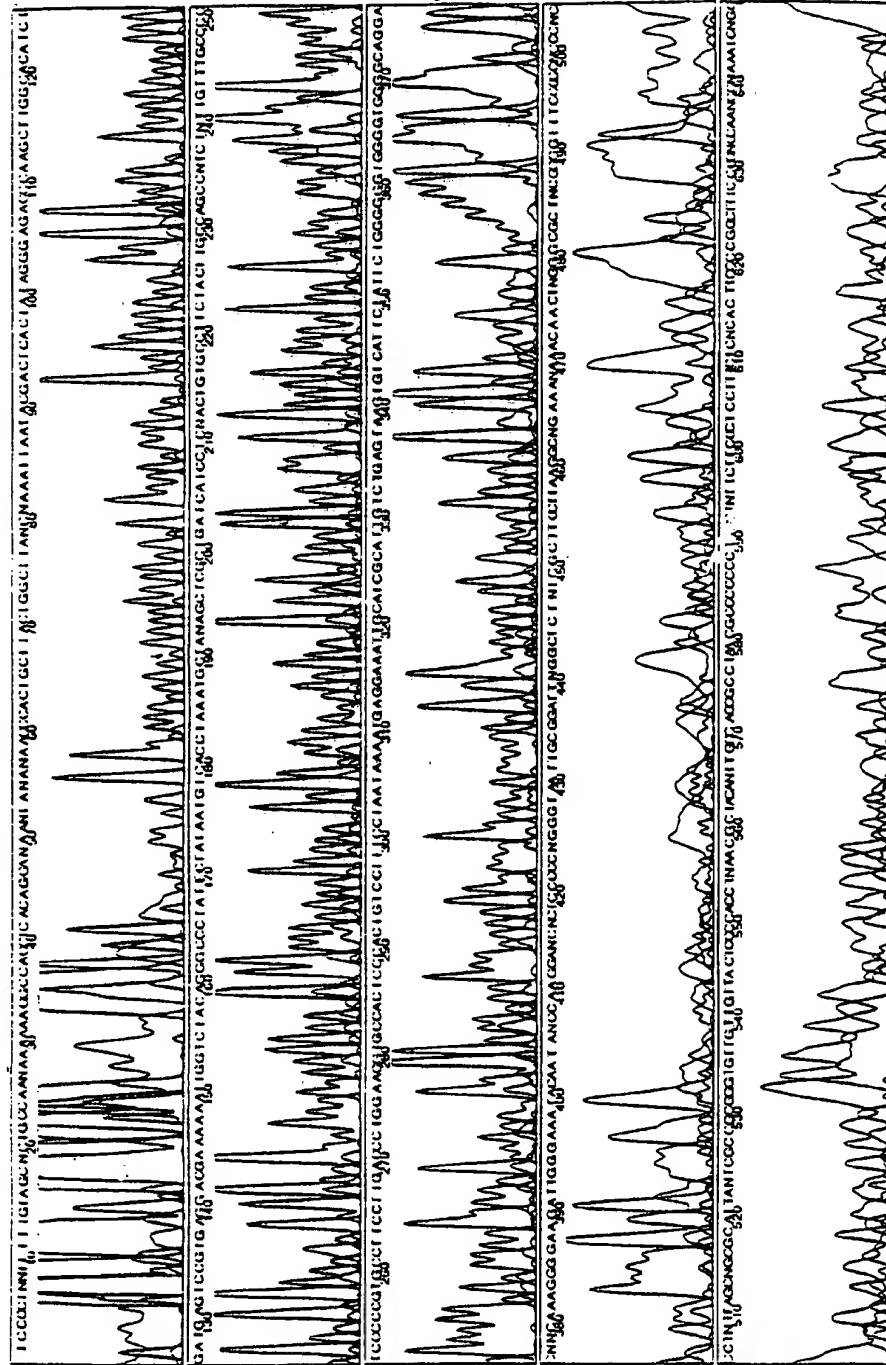
Seq. 4

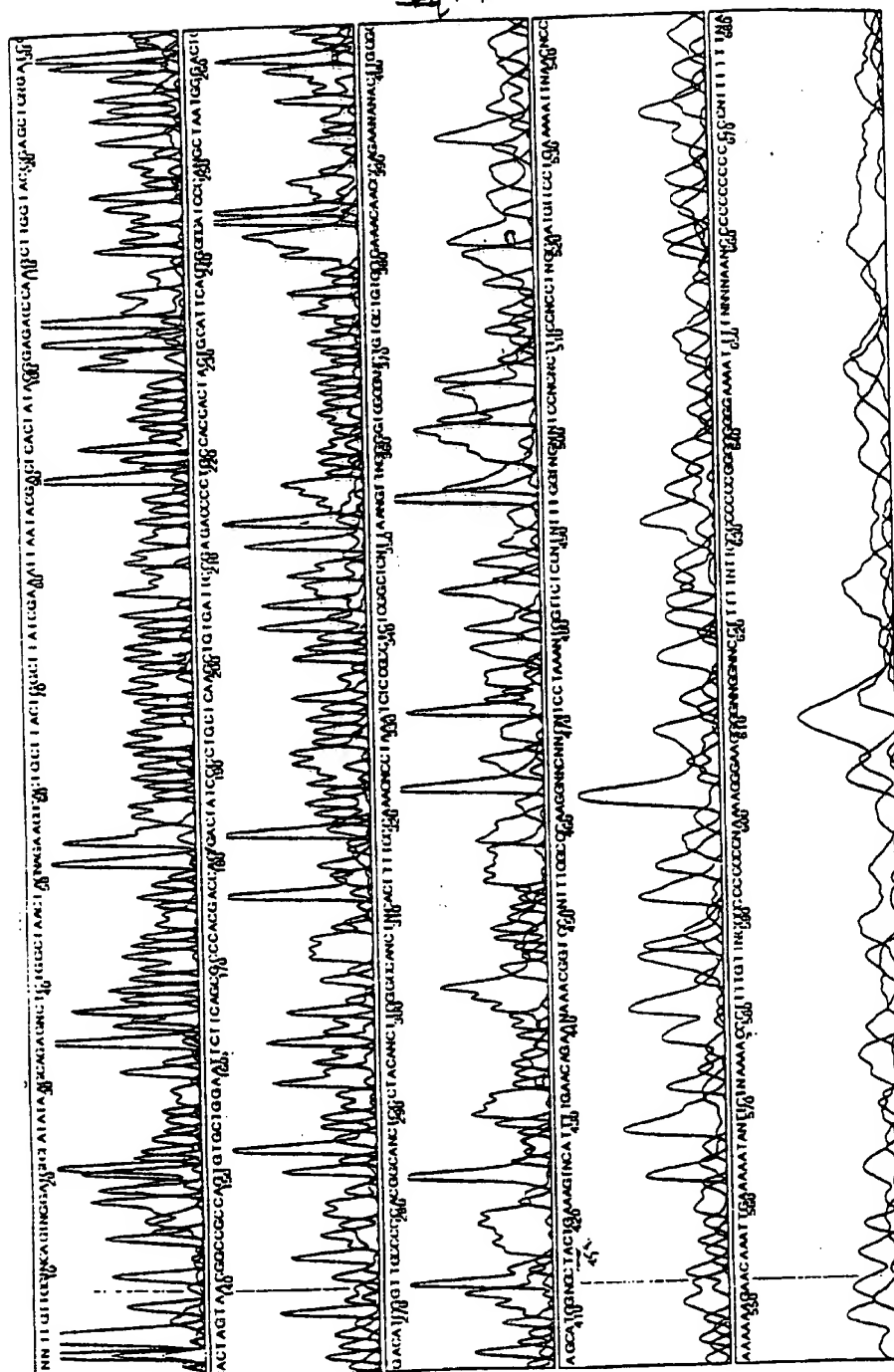


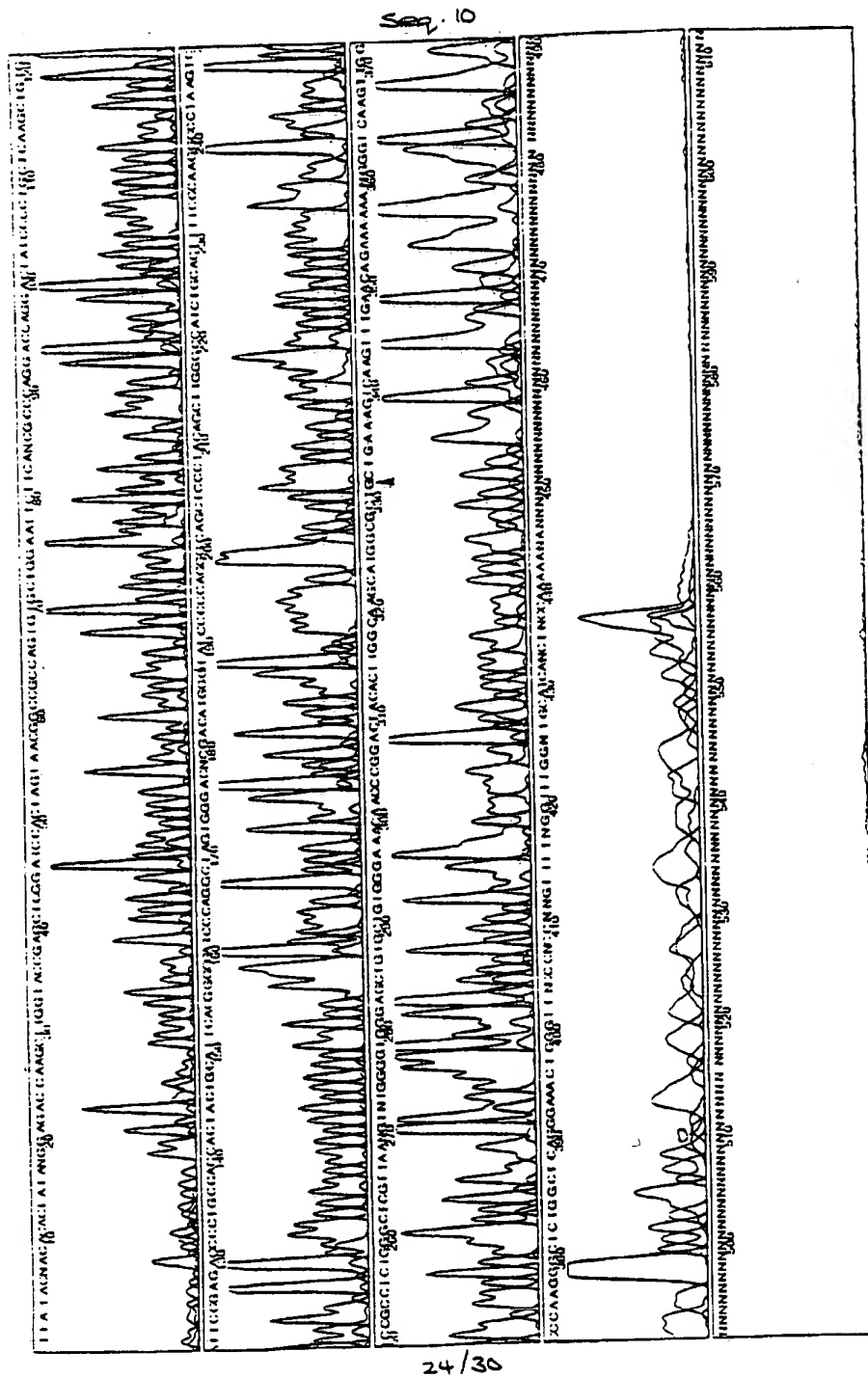


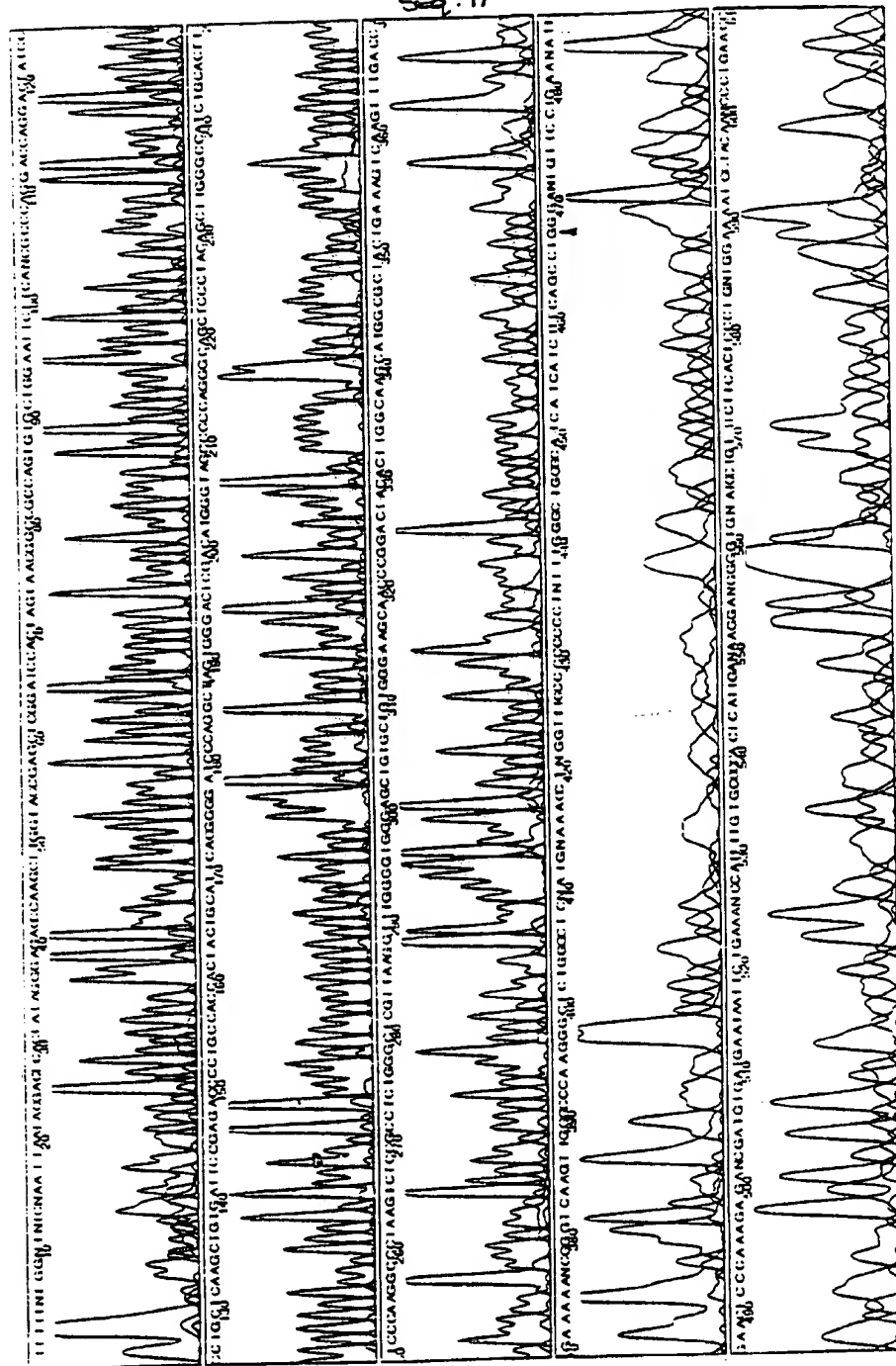


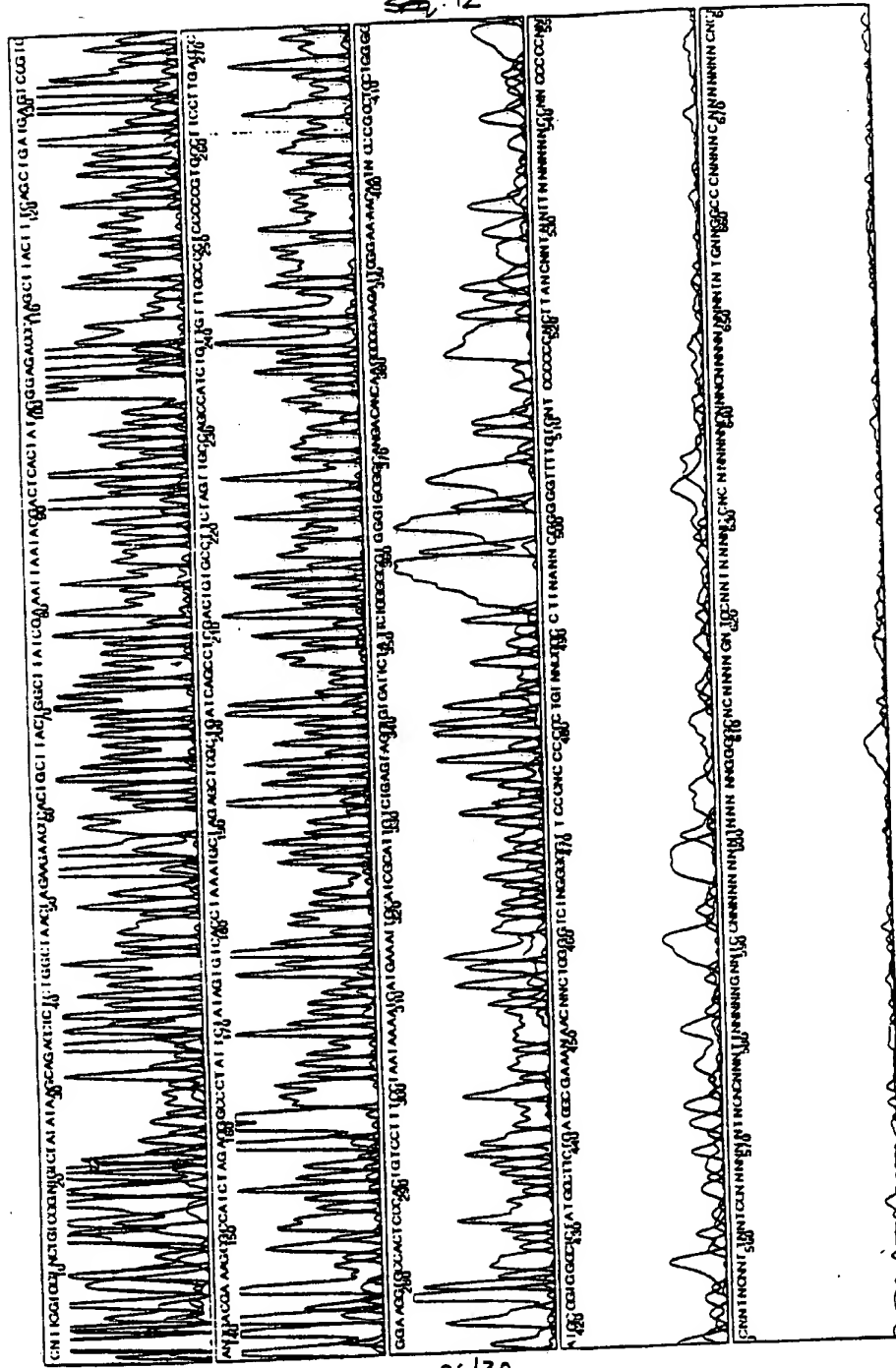


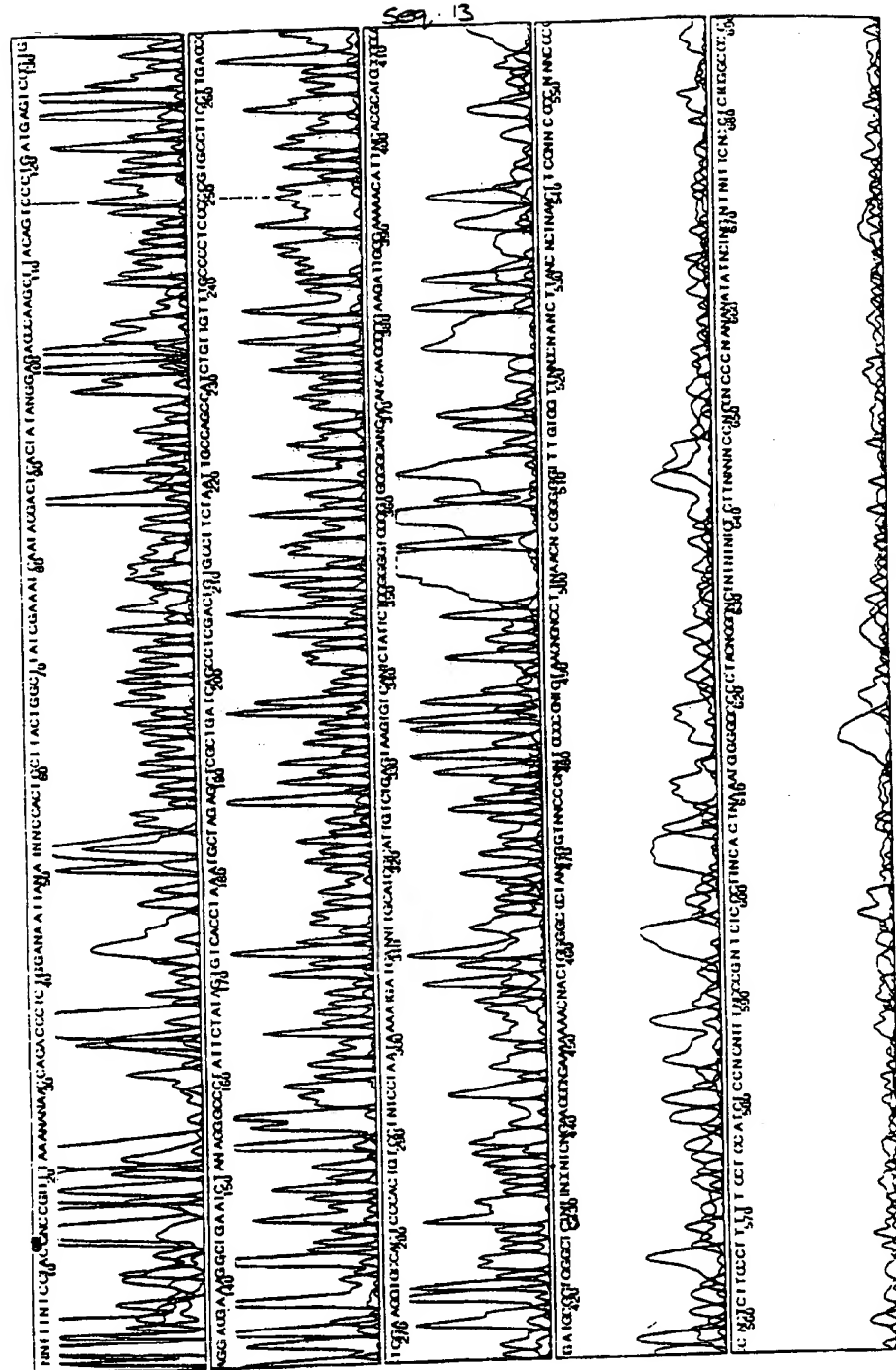


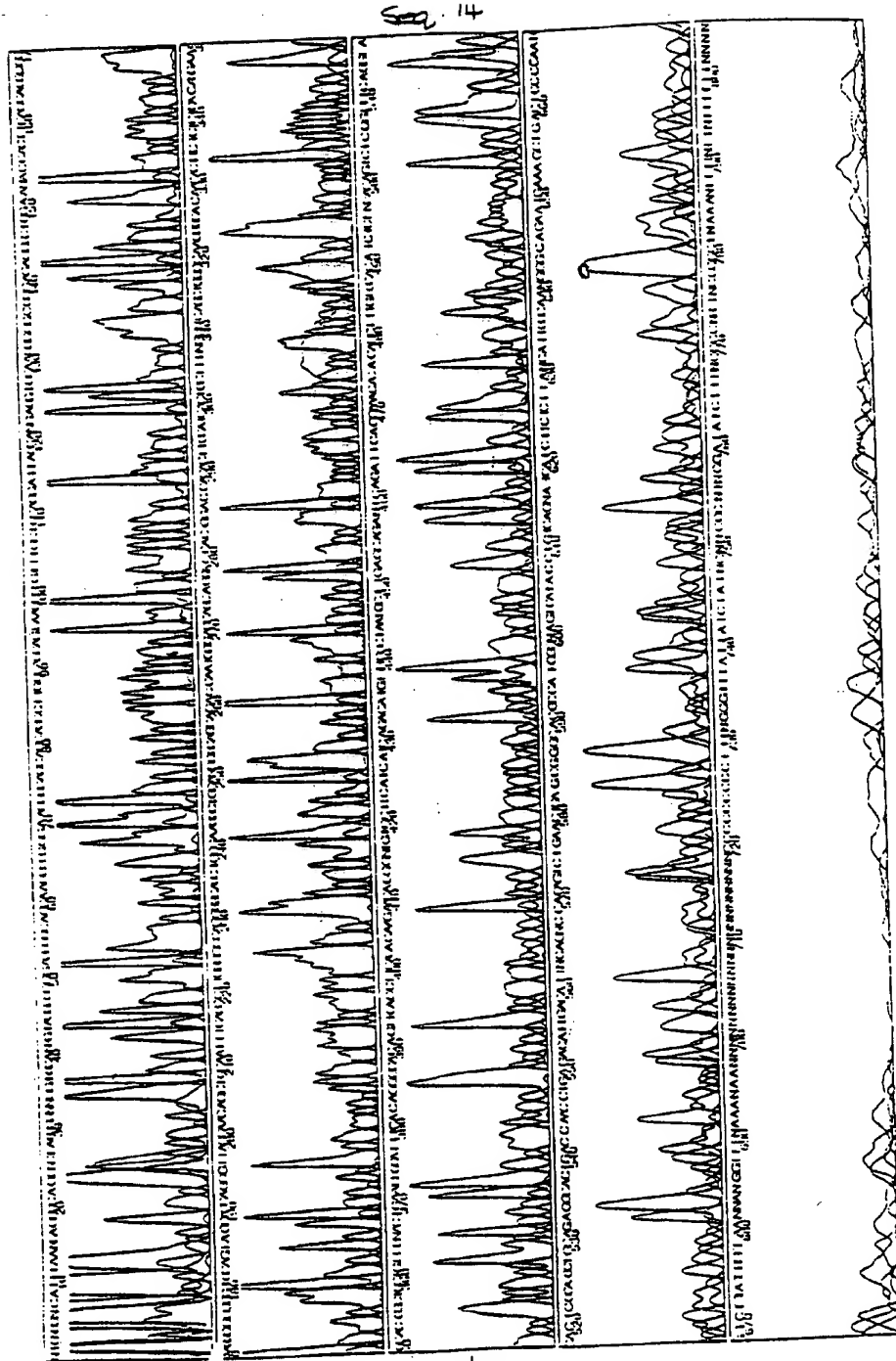


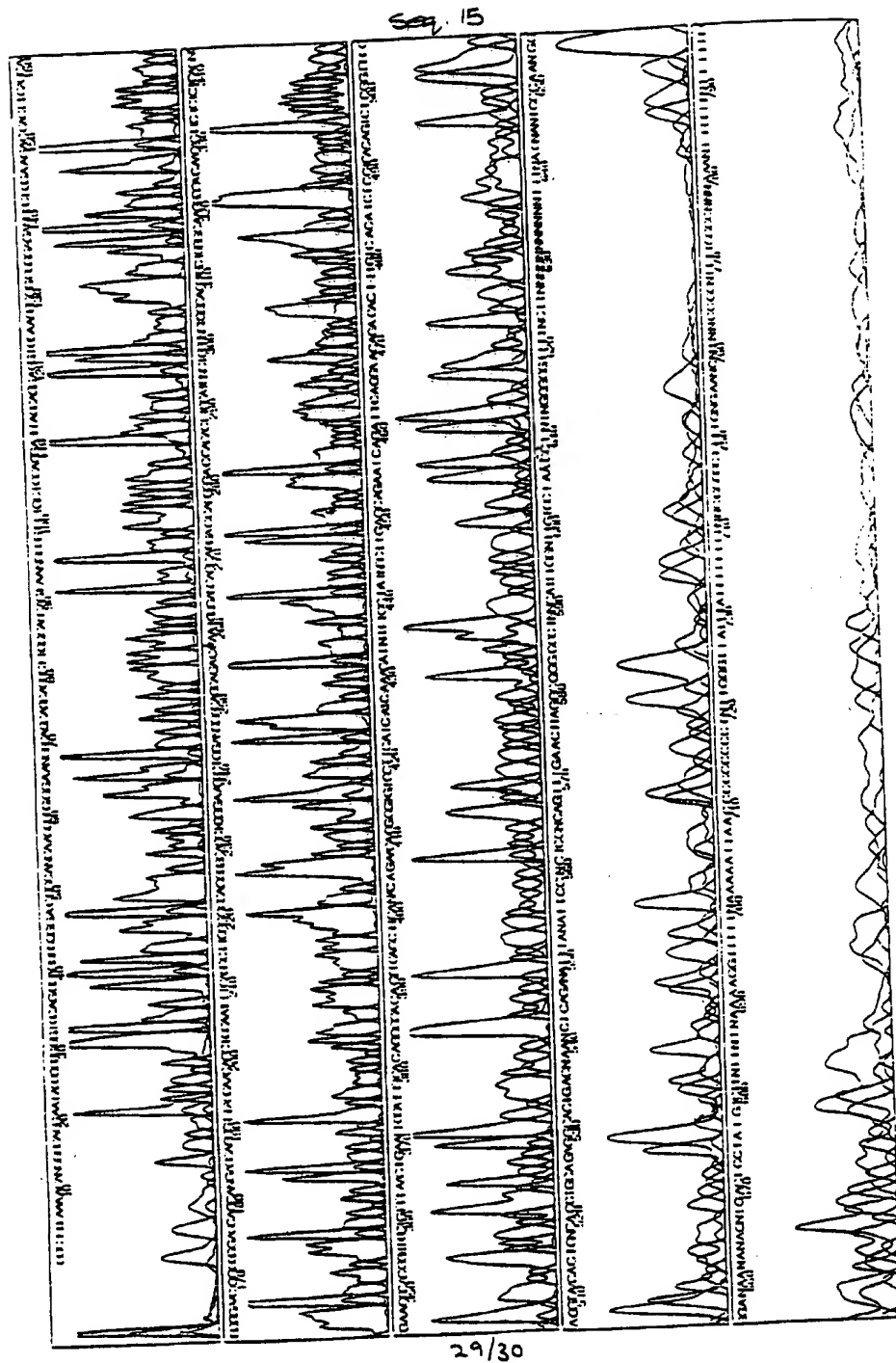


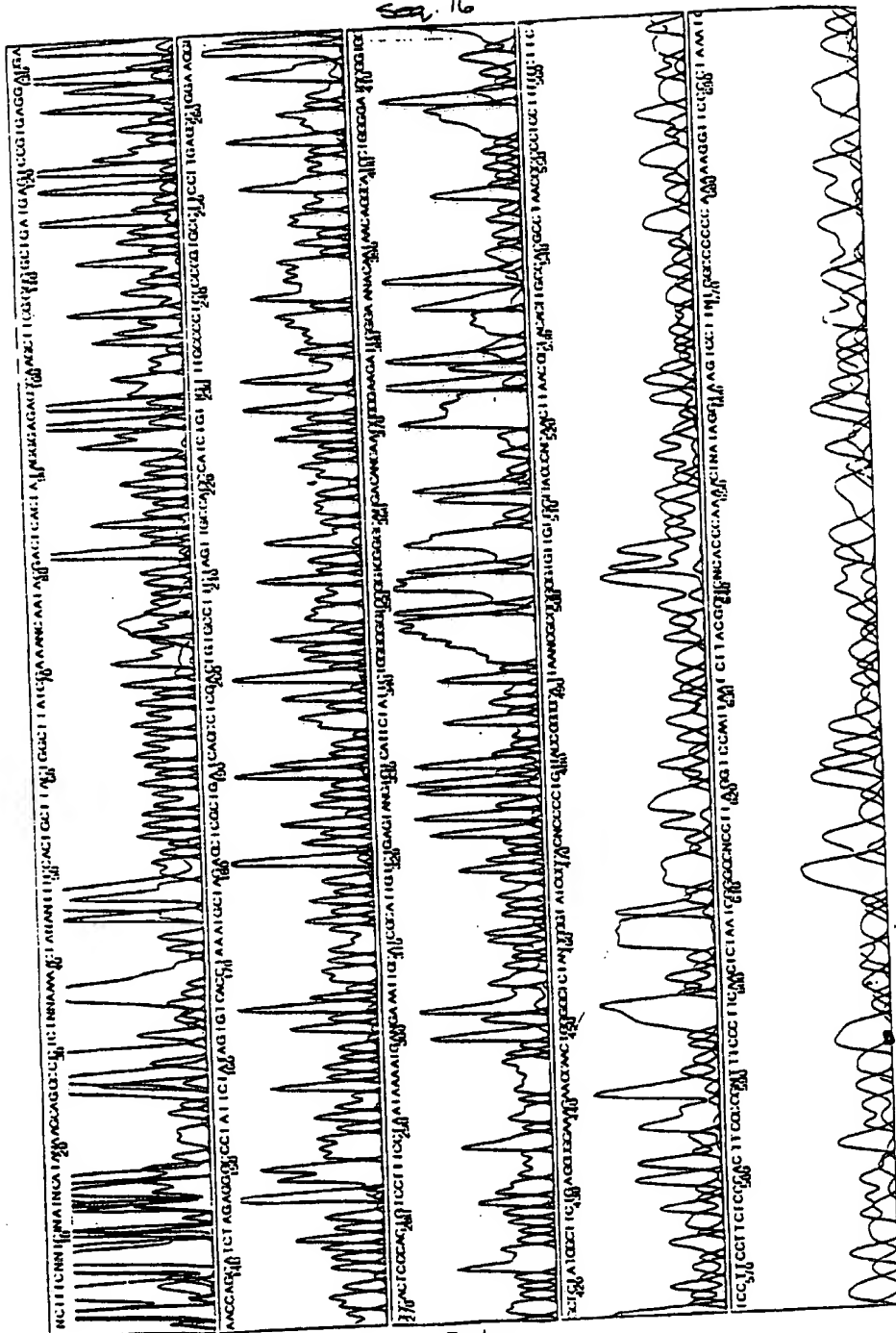












INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 97/00929

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/11 C12N9/00 A61K48/00 A61K31/70 C07H21/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A61K C07H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LEUKEMIA, vol. 8, no. SUPPL. 01, April 1994, pages 152-155, XP000652853 ROBINSON-BENION C ET AL: "GENE TRANSPLANTATION: COMBINED ANTISENSE INHIBITION AND GENE REPLACEMENT STRATEGIES"	1-3,7,8
Y	see the whole document	4,9
Y	HUMAN MOLECULAR GENETICS, (1995 SEP) 4 (9) 1597-602., XP002038178 HART S L ET AL: "The introduction of two silent mutations into a CFTR cDNA construct allows improved detection of exogenous mRNA in gene transfer experiments." see the whole document	4,9

-/--

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "A" document member of the same patent family

Date of the actual completion of the international search

20 August 1997

Date of mailing of the international search report

29.08.97

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
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Fax (+31-70) 340-3016

Authorized officer

Andres, S

INTERNATIONAL SEARCH REPORT

International Application No
PC1/GB 97/00929

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 475 623 A (UNIV CALIFORNIA) 18 March 1992	5,6
A	see page 7, line 37 - line 45 see page 8, line 6 - line 25	4,7-9
X	US 5 240 846 A (COLLINS FRANCIS S ET AL) 31 August 1993	5,6
	see column 3, line 3 - line 8 see column 6, line 58 - column 7, line 8 see example I	
A	WO 93 12257 A (HYBRITECH INC) 24 June 1993	4,9
	see page 8, line 12 - line 33 see page 32, line 30 - page 33, line 24 see page 36; example III	
A	WO 94 11494 A (UNIV JEFFERSON; PROCKOP DARWIN (US); COLIGE ALAIN (BE); BASERGA RE) 26 May 1994	
P,X	WO 97 11169 A (TRINITY COLLEGE DUBLIN ; FARRAR GWENYTH JANE (IE); HUMPHRIES PETER) 27 March 1997	1-3,7,8
	see page 6, line 25 - page 11, line 5 see examples	

INTERNATIONAL SEARCH REPORT

International application No.

PC1/GB 97/00929

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claim(s) 1-4, 7-9
is(are) directed to a method of treatment of the human/animal
body, the search has been carried out and based on the alleged
effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such
an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all
searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment
of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report
covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is
restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 97/00929

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		AU 8183694 A	23-02-95
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